Intranasal levels of lead as an exacerbation factor for allergic rhinitis in humans and mice

Huadong Xu, MPH[1,2], Nobutaka Ohgami, PhD[1,2], Masafumi Sakashita, MD, PhD[3], Kazuhiro Ogi, MD, PhD[3], Kazunori Hashimoto, PhD[1,2], Akira Tazaki, PhD[1,2], Keming Tong, BS[1,2], Masayo Aoki, MD, PhD[1,2], Shigeharu Fujieda, MD, PhD[3], Masashi Kato, MD, PhD[1,2,*]

[1]Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, 466-8550, Japan. [2]Voluntary Body for International Healthcare in Universities, Nagoya, Aichi, Japan. [3]Division of Otorhinolaryngology-Head & Neck Surgery, Department of Sensory and Locomotor Medicine, University of Fukui, Fukui, Japan.

***Corresponding author:** Masashi Kato, M.D., Ph.D., Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya 466-8550, Japan.
E-mail: katomasa@med.nagoya-u.ac.jp; Phone: 052-744-2124; Fax: 052-744-2126

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Abstract

Background: Air pollutants are suspected to affect pathological conditions of allergic rhinitis (AR).

Objective: After detecting lead (Pb) (375 μg/kg) in Japanese cedar pollen, the effects of intranasal exposure to Pb on symptoms of AR were investigated.

Methods: Pollen counts, subjective symptoms and Pb levels in nasal epithelial lining fluid (ELF) were investigated in 44 patients with Japanese cedar pollinosis and 57 controls from pre-season to season. Effects of intranasal exposure to Pb on symptoms were confirmed by using a mouse model of AR.

Results: Pb levels in ELF from patients were >40% higher than those in ELF from control subjects during the pollen season but not before the pollen season. Pb level in ELF was positively associated with pollen counts for the latest 4 days before visiting a hospital as well as scores of subjective symptoms. Intranasal exposure to Pb exacerbated symptoms in allergic mice, suggesting Pb as an exacerbation factor. Pb levels in ELF and nasal mucosa in Pb-exposed allergic mice were higher than those in Pb-exposed non-allergic mice, despite intranasally challenging the same amount of Pb. Since the increased Pb level in the nasal mucosa of Pb-exposed allergic mice was decreased after washing the nasal cavity, Pb on the surface of but not inside the nasal mucosa may have been a source of increased Pb level in ELF of allergic mice.

Conclusion: Increased nasal Pb level partially derived from pollen could exacerbate subjective symptoms of AR, indicating Pb as a novel hazardous air pollutant for AR.

Key messages

1. A higher level of lead in nasal epithelial lining fluid (ELF) from patients with pollinosis than that in ELF from control subjects was clinically obtained in season.
2. Correlations of lead levels in ELF with pollen counts as well as with severity of subjective symptoms were obtained.
3. The clinical results were partially confirmed by our animal study.

Capsule summary

An increased nasal level of lead partially derived from pollen may exacerbate allergic rhinitis in humans and mice. Thus, a novel association between pollinosis and an air pollutant (lead) was shown.

Key words: air pollutant, seasonal allergic rhinitis, exacerbation factor, intranasal exposure, lead, Japanese cedar pollen.

Abbreviations: AR, allergic rhinitis; JCP, Japanese cedar pollinosis; Pb, Lead; Cd, cadmium; Hg, mercury; ELF, nasal epithelial lining fluid; ICP-MS, inductively coupled plasma mass spectrometry; NLF, nasal lavage fluid; OVA, ovalbumin; LA-ICP-MS, laser ablation inductively coupled plasma mass spectrometry; Ag, antigen； BMI, body mass index; CI, confidence interval; OR, odds ratio; $PM_{2.5,}$ ambient fine particulate matter.

Introduction

The prevalence of seasonal allergic rhinitis, which is characterized by immunological reactions such as recruitment of eosinophils and release of cytokines, [1–3] has been rapidly increasing. Seasonal allergic rhinitis has become a global health problem because of its high worldwide prevalence (10-30%).[4] The prevalence of Japanese cedar pollinosis (JCP), a representative seasonal allergic rhinitis in Japan, has reached almost 50%,[2,5] and JCP is now called a folk disease in Japan.

Health disturbances caused by exposure to various elements including heavy metals have been widely recognized worldwide.[6-9] In fact, it has been shown that elements modulate immunological and inflammatory reactions.[10-13] Notably, levels of lead (Pb), cadmium (Cd) and mercury (Hg), which are representative air pollutants, have been reported to increase the risk of bronchial asthma in the general population via modulation of immunological potencies and airflow obstruction.[14–16] In the context of allergic rhinitis, studies have shown that pollen collected from highly air polluted areas has more allergic potency than that of pollen collected from unpolluted areas.[17,18] Since the sizes of air particles that cause bronchial asthma and allergic rhinitis[19] and the pathogenesis of these two diseases are different,[20,21] the contribution of air particles including Pb, Cd and Hg to allergic rhinitis should be investigated. However, there has been no clinical study showing the effects of heavy metals on allergic reaction in the nasal cavity of patients with seasonal allergic rhinitis before the season (pre-season) and during the season (season). Moreover, there has been no experimental study showing effects of intranasal exposure to heavy metals on pathological conditions of allergic rhinitis. Even the distribution of intranasally exposed heavy metals in nasal mucosa remains unknown.

In this study, inductively coupled plasma mass spectrometry (ICP-MS) analysis revealed for the first time the absolute values (means ± SD) of Pb (375 ± 517 μg/kg), Cd (31 ± 14 μg/kg) and Hg (10 ± 4 μg/kg) in Japanese cedar pollen (Figure S1). Based on the results, we first hypothesized that the heavy metals in pollens affect the pathogenesis of JCP. Then a clinical follow-up study using our previously reported methods[3,22,23] was conducted to investigate the contributions of Pb, Cd and Hg to the pathogenesis of seasonal allergic rhinitis under the condition of natural allergen exposure. To provide a more solid basis for a linkage between heavy metals and allergic rhinitis, the results obtained in human subjects were further confirmed by the results of a study using model mice with allergic rhinitis.

2. Methods

2.1. Pollen counts

Japanese cedar pollen grains were collected during the pre-season and season at the University of Fukui Hospital in Fukui, Japan by using a Durham sampler. After microscopically identifying Japanese cedar pollen grains that were dropped onto glass microscope slides, the number of pollen grains (/cm^2) was counted following our previously reported methods.[22,24]

2.2. Patients with JCP and control subjects in the clinical study

The clinical study was carried out under the condition of natural allergen exposure, which has been performed by previously reported methods with slight modifications.[3,23] A total of 103 subjects were recruited in Fukui City, Japan. Since the pollen dispersal period is typically between February and April,[2,22] a follow-up study was conducted from January (pre-season) to April (season) as shown in

Figure S2. At baseline, we collected information on demographic characteristics such as age, sex, smoking and personal medical history. All of the subjects had no other respiratory or immune diseases or any other severe diseases during the study. After exclusion of 2 subjects (1.9%) due to incomplete data in the follow-up questionnaire, the subjects were divided into a group of 57 control subjects (controls) and a group of 44 patients who had a history of JCP and positive anti-Japanese cedar pollen-specific IgE (≥ 0.35 IU/mL) determined by ImmunoCAP (patients), following previous papers.[25,26] Each subjective symptom including sneezing, nasal discharge and nasal blockage in season was recorded from a questionnaire. Total subjective symptoms including sneezing, nasal discharge and nasal blockage were scored by a previously validated method.[2,27]

2.3. Measurements of heavy metal concentrations in human samples

Samples of nasal lavage fluid (NLF), serum, saliva and urine were collected in pre-season and season from subjects with JCP (n=44) and control subjects without JCP (n=57) using previously described methods with slight modifications.[1,3] Pure water (10 mL) was used to collect NLF in this study. All of the samples were rapidly stored at −80°C. Japanese cedar pollen grains collected from 3 different locations were purchased from 3 different companies (Biostir Inc., Osaka; ITEA Inc., Tokyo and Yamizo Pollen Research Group, Ibaraki). Pollens of silver birch pollen and orchard grass in Sweden and pollens of ragweed in China were obtained from a company (ITEA Inc, Tokyo). Pb, Cd and Hg concentrations in human samples (NLF, saliva and urine) and in Japanese cedar pollen were measured by an inductively coupled plasma-mass spectrometer (ICP-MS, 7500cx, Agilent Technologies, Inc) using the method previously described.[28,29] Levels of urea in sera and nasal

155 epithelial lining fluid (ELF) are constant regardless of allergic reactions, indicating
156 that urea concentration could be a reliable dilution factor.[3,30] As in our previous
157 studies,[3,22] concentrations of Pb, Cd and Hg in ELF were adjusted by urea
158 concentrations, according to the following formula:

159 $$\text{Heavy metal levels in ELF samples} = \text{Heavy metal levels in NLF} \times \frac{\text{Urea in serum}}{\text{Urea in NLF}}.$$

160 In accordance with previous studies,[31,32] urinary levels of Pb, Cd and Hg were
161 presented as the concentrations equal to one gram of creatinine according to the
162 following formula:

163 $$\text{Heavy metal levels in urine samples} = \frac{\text{Urinary heavy metal concentration}}{\text{Urinary creatinine concentration}}.$$

164

165 **2.4. Model mice for allergic rhinitis**

166 Ovalbumin (OVA, Fujifilm Wako Pure Chemical Co., Osaka, Japan) was used
167 as an antigen to develop model mice for allergic rhinitis as shown previously.[33,34]
168 Levels of Pb, Cd and Hg in OVA were undetectably low by our ICP-MS analysis.
169 Female BALB/c mice obtained from Japan SLC Inc. (Hamamatsu, Japan) were
170 maintained in specific pathogen-free conditions and used at 6 weeks of age. The
171 mice were divided into four groups including three groups treated with OVA and/or
172 Pb and one control group without treatment. Briefly, mice were treated by
173 intraperitoneal injection of 25 μg of antigen plus 1.5 mg of aluminum hydroxide
174 (Fujifilm Wako Pure Chemical Corp, Osaka, Japan) in 200 μL of saline or the same
175 volume of saline on days 0, 7 and 14. From day 21, mice were intranasally
176 challenged by saline, lead nitrate (Kanto Chemical Co., Saitama, Japan), 500 μg
177 antigen and 500 μg antigen plus lead nitrate in 20 μL of saline for 7 consecutive
178 days. The Pb dose used in this study was 1.75 μg (about 2.80 μg lead nitrate), which

corresponds to the maximum level of Pb detected in ELF from patients with JCP in this study.

Frequencies of sneezing and nasal rubbing were investigated 10 minutes after the final intranasal challenge of antigen and/or Pb on day 27. NLF and nasal tissue were collected 24 hours after the final intranasal challenge. Conversion of Pb concentrations from NLF to ELF in mice was performed by the method used in our clinical study. After developing paraffin blocks of murine nasal tissues with decalcification, 5-μm-thick sections were developed and stained with hematoxylin and eosin (HE) and with Alcian blue-periodic acid-Schiff (PAS) staining for analysis of goblet cells and mucins, respectively. After developing frozen blocks of murine nasal tissues without decalcification, the blocks were sliced into 20-μm-thick sections and mounted onto glass slides. The Pb distributions in nasal tissue sections were determined by using a laser ablation system (LSX-213 G2+, Teledyne CETAC Technologies, Omaha, NE, USA) equipped with an ICP-MS (ICP-MS, 7700x, Agilent Technologies, Inc) instrument (LA-ICP-MS). The nasal tissue sections were ablated by a focused laser beam (energy of 10.2 J/cm^2, spot size of 10 μm, repetition frequency of 10 Hz and scan speed of 10 μm/s) in our LA-ICP-MS scanning system. The signal intensities of ^{208}Pb and ^{31}P were normalized by endogenous ^{13}C signal intensity (^{208}Pb/^{13}C and ^{31}P/^{13}C) and distribution maps were developed using iQuant2+ software (Tokyo Institute of Technology, Japan).[35] Consistent results were confirmed by analysis of three or more nasal tissues per group, and representative results are presented.

2.5. Ethical permission

This study was approved by ethical committees following the regulations of the Japanese government in Nagoya University (approval No. 2014-0034) and Fukui University (approval No. 20150092). All subjects gave written informed consent. Animal experiments, all of which were performed only in Nagoya University, were approved by the Institutional Animal Care and Use Committee in Nagoya University (approval number: 31233) that was organized following the Japanese government regulations for animal experiments.

2.6. Statistical analysis in the clinical and animal studies

Student's *t*-test, the Mann-Whitney *U* test and the chi-square test were used to analyze differences between subjects with allergic rhinitis and control subjects as described previously.[36,37] One-way ANOVA followed by the LSD test was used in the mouse study. The differences in changes of Pb concentrations in patients from pre-season to season relative to those in the control subjects using generalized linear mixed-effects models were estimated by the previously reported method with slight modification.[38] We calculated Δconcentration (Pb concentration during season - Pb concentration during pre-season) and used it in the models to control for the impact of different baseline levels of Pb during pre-season. All models for changes of Pb concentrations in human samples were adjusted for fixed-effect covariates including age, sex, BMI and smoking history. Since all of the participants lived in similar areas with similar socio-economic status in Fukui Prefecture, it was not necessary to adjust potential environmental confounding such as traffic pollution conditions.

Spearman's rank correlation analysis was first used to evaluate the univariate associations between Pb concentrations in human samples and each nasal symptom score.[39] The correlation between incidence of each subjective symptom

and Pb concentrations in biological samples was then assessed using logistic regression models adjusted for age, sex, BMI and smoking history after Pb levels had been log-transformed. Physical activity and sleep time are also thought to be associated with allergic rhinitis symptoms.[40] Thus, we further adjusted for physical exercise days (≤2 days/week and >2 days/week) and sleep time.

In order to investigate the potential effects of Japanese cedar pollen on Pb concentrations, the relationship between Pb levels in biological samples and cedar pollen counts was analyzed using generalized linear mixed-effects models. The lagged relationship between Pb levels in biological samples on day 0 when participants visited a hospital for collection of biological samples and Japanese cedar pollen counts 0-9 days before visiting the hospital was assessed using a previously described method.[41] In this analysis, Pb levels in ELF, saliva and urine samples of each subject were associated with the corresponding pollen counts. Age, sex, BMI and smoking history were included as fixed-effect factors to control for subject variability.

All statistical analyses in this study were performed by IBM SPSS Statistics (version 25.0) and R Programming Language (version 3.4.3). A two-sided *P* value less than 0.05 was taken as statistical significance.

3. Results

3.1. Basic information on participants in the clinical study

The basic characteristics of the participants are shown in Table 1. The subjects were 57 control subjects including 31 females and 26 males (mean age: 41.5 ± 10.3 years) and 44 patients with JCP including 25 females and 19 males (mean age: 39.8 ± 10.3 years). There were no significant differences in age, sex, BMI, smoking

history, physical activity time and sleep time between patients and control subjects (Table 1). The patients had a significantly ($P < 0.001$) higher mean baseline level of Japanese cedar pollen-specific IgE (11.3 ± 15.9 IU/mL) than that in the control subjects (0.1 ± 0.1 IU/mL). Scores for sneezing, nasal discharge, nasal blockage and total subjective symptoms in the patients were significantly ($P < 0.001$) higher than those in the control subjects in season (Figure S3).

3.2. Pb levels in participants in pre-season and season in the clinical study

The Pb levels in ELF from patients were significantly higher than those from controls in season, while the Pb levels in ELF were comparable in patients and control subjects in pre-season (Figure 1A). There were no significant differences in Pb levels in saliva and urine samples between control subjects and patients in pre-season and season (Figure 1B, C). On the other hand, Cd levels in ELF and saliva samples and Hg levels in ELF, saliva and urine samples were undetectably low in the participants in both pre-season and season (Table S1). There was no significant difference in urinary Cd levels between patients and control subjects (Table S1). Therefore, analyses focusing on Pb were then performed.

Repeated-measures generalized mixed model analyses were performed to assess changes in Pb levels in patients from pre-season to season relative to those in control subjects with adjustment for age, sex, BMI and smoking history (Figure 1D). The results of multivariate analysis showed a 67.93% [95% confidence interval (CI): 4.29%~131.56%, $P = 0.037$] greater increase of Pb level in ELF from patients than the change of Pb level in ELF from control subjects from pre-season to season. The relative changes of Pb in saliva (-24.82%, 95%CI: -80.75%~64.14%, $P = 0.821$)

and urine (-24.82%, 95%CI: -108.01%~58.30%, *P* = 0.554) from pre-season to season were not significantly different between patients and controls.

3.3. Correlations between nasal symptoms and Pb levels in patients in season

Correlations between each nasal symptom score and Pb levels in human samples from patients in season were next investigated. Spearman's correlation analysis revealed significant associations between Pb levels in ELF and scores for sneezing (*r* = 0.22, *P* = 0.026), nasal blockage (*r* = 0.34, *P* = 0.001) and total subject symptoms (*r* = 0.25, *P* = 0.013), while the score for nasal discharge was not associated with Pb levels in ELF samples (Table S2). Log-transformed Pb levels in ELF remained positively associated with incidences of sneezing (odds ratio [OR] = 5.27, 95% CI: 1.37~20.28, *P* = 0.016), nasal blockage (OR = 7.67, 95% CI: 1.69~34.88, *P* = 0.008) and total subjective symptoms (OR = 7.28, 95% CI: 1.88~28.20, *P* = 0.004) in multivariable models adjusting for age, sex, BMI, smoking history, sleep time and physical activity (Figure 2A). There was no significant correlation between each nasal symptom and Pb levels in saliva or urine (Figure 2B, C).

The correlations between the number of Japanese cedar pollen grains and Pb levels in human samples in season were then investigated. There were significant positive correlations between pollen counts for 4 days before visiting a hospital and Pb levels in ELF (Table 2), while there was no significant correlation between pollen counts and Pb levels in saliva and urine (Table S3). These results indicate a positive dose-response between pollen counts and Pb level in ELF but not Pb level in saliva or urine.

3.4. Effect of Pb on allergic rhinitis in model mice

In order to confirm the results of our clinical study, animal studies using mice with allergic rhinitis and mice without allergic rhinitis were conducted with or without nasal challenge of Pb and/or the antigen (Figure 3A). Levels of symptoms (sneezing and nose rubbing) in mice without allergic rhinitis just after nasal challenge of Pb were comparable to those just after nasal challenge of the solvent of Pb (control) (Figure 3B, C). However, those in mice with allergic rhinitis just after nasal challenge of the antigen and Pb were significantly higher than those just after nasal challenge of the antigen (Figure 3B, C), indicating that nasal exposure to Pb exacerbated nasal symptom of the mice with allergic rhinitis.

The concentration of Pb in ELF and distribution of Pb in the nasal cavity of mice with and those without allergic rhinitis were finally determined with ICP-MS (Figure 3D) and LA-ICP-MS (Figure 3E), respectively. In control mice without allergic rhinitis, Pb level in ELF 24 hours after the nasal challenge of Pb was comparable to that 24 hours after the nasal challenge of the solvent of Pb (Figure 3D). In mice with allergic rhinitis, the mean level of Pb 24 hours after nasal challenge of the antigen plus Pb was 8.4-fold higher than that 24 hours after nasal challenge of the antigen (Figure 3D). More importantly, the mean level of Pb in ELF from mice with allergic rhinitis 24 hours after nasal challenge of the antigen plus Pb was 4.3-fold higher than that in ELF from control mice without allergic rhinitis 24 hours after nasal challenge of Pb (Figure 3D). Correspondingly, the Pb level in nasal mucosa from mice with allergic rhinitis 24 hours after nasal challenge of the antigen plus Pb was higher than that nasal mucosa from mice without allergic rhinitis 24 hours after nasal challenge of Pb in the condition before washing their nasal cavities (Figure 3E). These results suggest that intranasally challenged Pb remained at a high level in the nasal cavity

24 hours after nasal challenge of Pb in allergic mice but not in non-allergic mice despite the fact that allergic mice and non-allergic mice were intranasally exposed to the same amounts of Pb.

4. Discussion

Our ICP-MS analysis showed that the mean level of Pb in Japanese cedar pollen was more than 10-fold higher than the mean levels of Cd and Hg (Figure S1). Our longitudinal clinical study showed undetectably low levels of Cd and Hg except for Cd level in urine samples in the participants throughout pre-season and season (Table S1). Moreover, there was no significant difference between urinary Cd levels in patients and control subjects (Table S1). On the other hand, our clinical study showed an increased level of Pb in ELF but not in saliva or urine from patients with JCP in season. Positive correlations between subjective symptom scores and Pb levels in ELF but not in saliva and urine were also found. These results suggest that the level of Pb, but not the Cd or Hg level, in the nasal cavity, a central site for the development of allergic inflammation, affects symptoms of allergic rhinitis in season. Therefore, further study was conducted with focus on intranasal Pb. Our clinical study showed that pollen counts for 4 days before visiting a hospital were significantly correlated with Pb level in ELF but not with Pb level in saliva or urine in participants (Table S3). Our results suggest that increased Pb level in ELF is partially derived from pollen. Thus, our clinical study suggests not only a potential source of intranasal Pb but also a potential role of intranasal Pb as an exacerbator for activated allergic rhinitis in season.

Correspondingly, our experimental study with model mice for allergic rhinitis indicated that intranasal exposure to Pb could exacerbate nasal symptoms of

activated allergic rhinitis provoked by an antigen. The combined results of our clinical and animal studies provided more solid evidence that intranasal exposure to Pb can exacerbate symptoms of allergic rhinitis.

Our results give rise to the question of why Pb levels in ELF from patients with allergic rhinitis were higher than the levels in ELF from control subjects without allergic rhinitis despite the fact that all of the subjects living in the similar areas should have been exposed to comparable amounts of pollen including Pb. In our animal study in which mice with allergic rhinitis and mice without allergic rhinitis were intranasally exposed to the same amounts of Pb, Pb levels in ELF from mice with allergic rhinitis were higher than the levels in ELF from control mice without allergic rhinitis. The combined results of our clinical and animal studies indicated the possibility of higher Pb levels in ELF of individuals with activated allergic rhinitis than in ELF of individuals without allergic rhinitis is possible even if the amounts of Pb exposure are the same.

Our LA-ICP-MS analysis newly showed that Pb level in nasal mucosa from Pb-challenged mice with activated allergic rhinitis remained at a high level 24 hours after intranasal challenge of Pb. The increased level of Pb in nasal mucosa of the Pb-challenged allergic mice was decreased after washing the nasal cavity and was comparable to that in nasal mucosa of the Pb-challenged non-allergic mice (Figure S4). Furthermore, the relative numbers of goblet cells (Figure S5A, B) and mucins-secreting goblet cells (Figure S5A, C) in nasal mucosa from allergic mice challenged with the antigen plus Pb were larger than those in nasal mucosa from allergic mice challenged only with the antigen. These results suggested that intranasally exposed Pb non-specifically trapped by sticky mucin on the surface of but not inside nasal

mucosa in mice with activated allergic rhinitis could be a source of increased Pb level in ELF of the mice.

A few limitations should be considered in this study. First, we speculated that pollen is one of the primary pollutant sources of Pb in ELF based on the correlation between a representative pollen count on each day and Pb levels in ELF. Although determination of the Pb level in pollen to which each patient had been exposed day by day would have been ideal, the determination from a practical/logistical standpoint is difficult. Second, there was no information for post-season, though characteristic pathological conditions have been reported in patients with allergic rhinitis in post-season.[3,42,43] Third, patients with allergic rhinitis are continually exposed to air pollutants with Pb for more than 5 weeks in season, while once-a-day exposure to Pb for 7 days was performed in a mouse model with allergic rhinitis. Thus, the patterns of intranasal exposure to Pb in patients and mice are different. On the other hand, the median Pb level in ELF (27.2 μg/L) in patients with allergic rhinitis in season (Figure 1A) was approximately comparable with that (13.7 μg/L) in mice with allergic rhinitis treated with 1.75 μg Pb (Figure 3D). The intranasal treatment with 1.75 μg Pb in mice seems to be physiologically reasonable.

There have been a variety of quantitative allergotoxicologic studies[44-46] in which the effects of total suspended particles including $PM_{2.5}$ on allergic rhinitis were examined. Although there have been qualitative allergotoxicologic studies[46,47] on the effects of components in total suspended particles on allergic rhinitis, the amount of information about the components that exacerbate allergic rhinitis is insufficient. Our clinical and animal studies qualitatively demonstrated for the first time that increased intranasal Pb level partially derived from Japanese cedar pollen can exacerbate symptoms of allergic rhinitis. Pollinosis caused by various cedars has been observed

401 all over the world.[48,49] There are high homologies of sequence (70%-90%) for major
402 antigens of cedar pollens in the world.[49] Table S4 shows approximately comparable
403 (mean levels: 143-2,278 μg/kg) levels of Pb in allergic rhinitis-inducing pollens and
404 bee pollens in European and Asian countries compared to that in Japanese cedar
405 pollen. In addition, pollution of pollens with Pb in the USA and Canada was
406 suggested from results for pollution of honey with Pb.[50,51] Taken together, a
407 contribution of Pb adhering to pollen (an air pollutant) to allergic rhinitis could be a
408 worldwide issue. Further allergotoxicologic studies are needed to clarify the
409 molecular mechanism of Pb-mediated exacerbation of allergic rhinitis and to
410 qualitatively identify other exacerbation factors for allergic rhinitis.

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Figure legends

Figure 1. Characterization of Pb in Japanese cedar pollinosis. (A-C) Pb levels in nasal epithelial lining fluid (ELF) (A), saliva (B) and urine (C) samples before the Japanese cedar pollen season (pre-season) and in the Japanese cedar pollen season (season) are presented. Concentrations (medians) of Pb in ELF, saliva and urine samples from control subjects (n=57, open squares) and patients with Japanese cedar pollinosis (n=44, closed squares) during pre-season and season are presented. The *P* value was calculated by the Mann-Whitney *U* test. (D) Relative percent changes of Pb concentrations from pre-season to season in patients compared to changes in controls are presented. The change is a relative percent change determined by using change of Pb levels in controls as the denominator and change of Pb levels in patients as the numerator. Generalized linear mixed-effects models were adjusted for fixed-effect covariates including age, sex, BMI and smoking status. Line bars indicate 95% confidence intervals (CIs).

Figure 2. Correlations between subjective symptoms and Pb levels in human samples. (A-C) Adjusted odds ratios for associations between subjective symptoms and log-transformed Pb levels in ELF (A), saliva (B) and urine (C) are presented. Logistic regression models were adjusted for baseline covariates including age, sex, BMI, smoking history, sleep time and physical activity. Line bars indicate 95% CIs.

Figure 3. Pb exacerbates nasal symptoms in a mouse model of allergic rhinitis. (A-E) The protocol of the mouse experiment is presented. After mice had been sensitized with an intraperitoneal injection of an antigen (Ag) or a solvent of the Ag

(saline), they were intranasally challenged with saline (Ctrl), Pb, Ag or Ag plus Pb (Ag+Pb) (A). Nasal symptoms (B, C), Pb level in ELF (D) and distribution of Pb in nasal mucosa of nasoturbinate in the condition before washing (E) in each group (n=7-10) are presented. Significant differences were analyzed by ANOVA followed by the LSD test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; N.S., not significant). Scale bar, 100 μm.

Table 1. Basic information of all participants

Variables	Control (n = 57)	Patient (n = 44)
Age (years)[a]	41.5±10.3	39.8±10.3
Sex[b]		
Male	26 (45.6%)	19 (43.2%)
Female	31 (54.4%)	25 (56.8%)
BMI (kg/m^2)[a]	22.0±3.3	21.7±3.0
Smoker[b]		
No	36 (63.2%)	34 (77.3%)
Yes	21 (36.8%)	10 (22.7%)
Sleep time (hours/day)[a]	6.1±0.9	6.4±0.9
Physical activity time[b]		
≤2 days/week	45 (78.9%)	33 (75.0%)
>2days/week	12 (21.1%)	11 (25.0%)
Japanese cedar pollen-specific IgE (IU/mL)[c]	0.1±0.1	11.3±15.9***

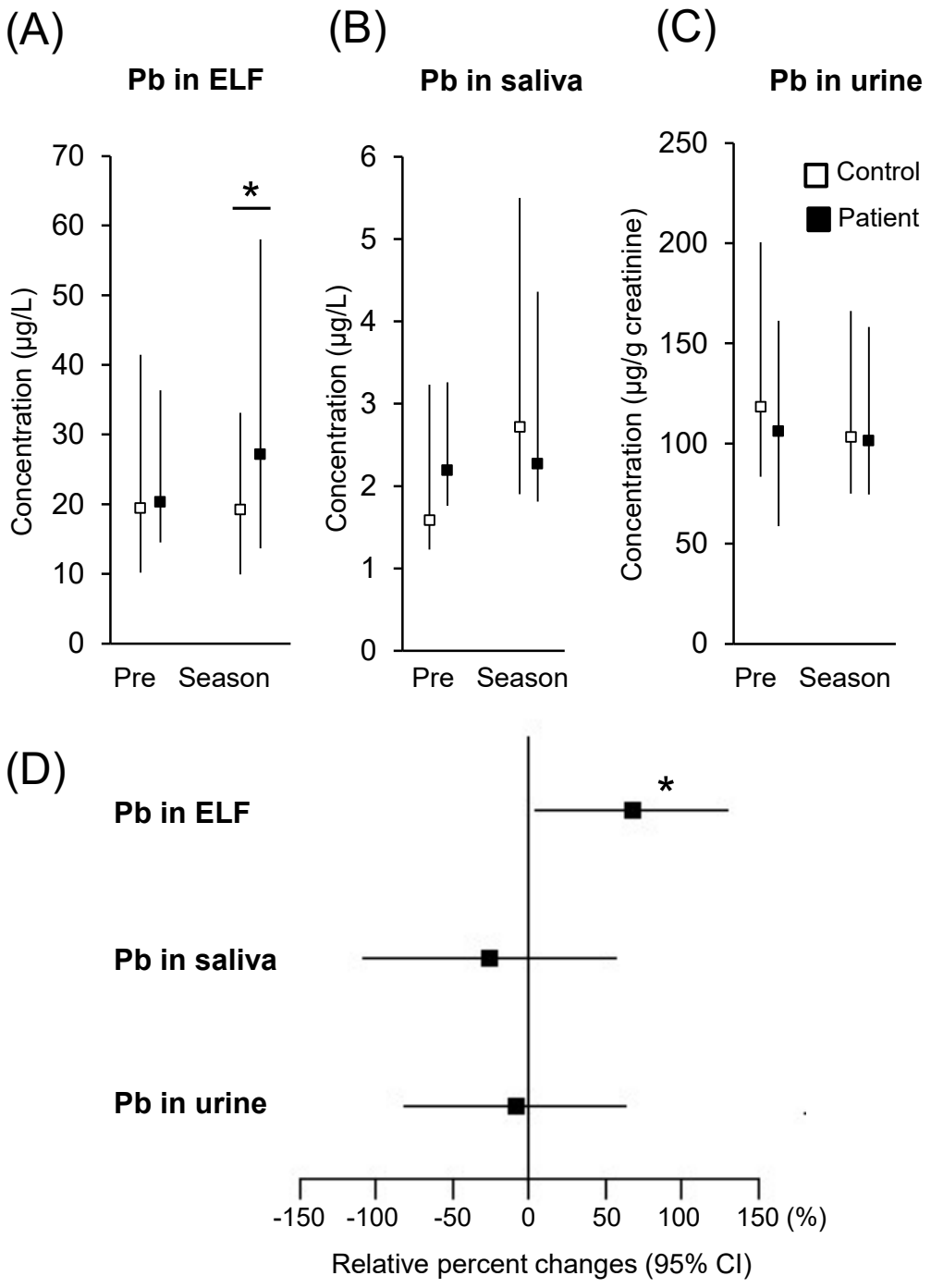
BMI = body mass index. Data are expressed as means± SD for continuous variables and as numbers (percentages) for categorical variables.

P values were calculated by [a]Student's t-test, [b]chi-squared test and [c]Mann-Whitney *U* test, respectively. ***$P < 0.001$.

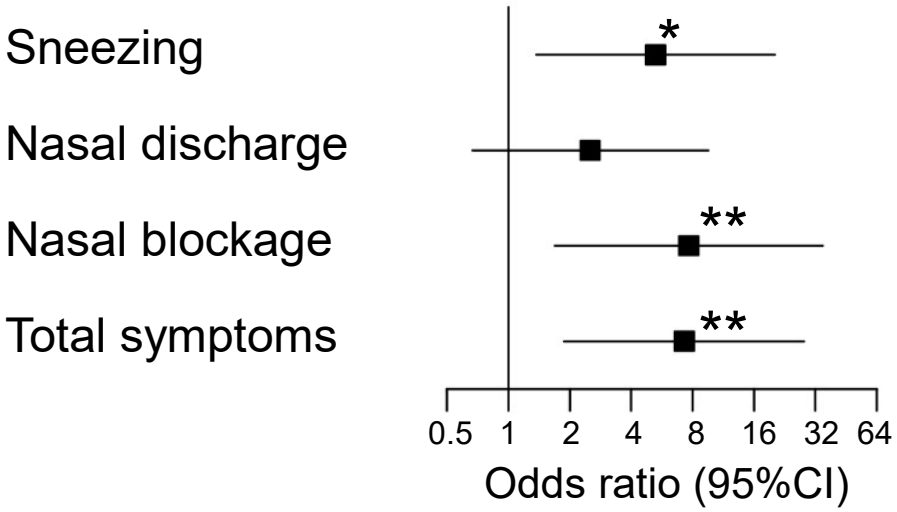
Table 2. Correlations between Pb levels in ELF and Japanese cedar pollen counts

Time lag	β	95% CI	*P*-Value
9 days	-0.04	(-0.09, 0.01)	0.116
7 days	-0.06	(-0.12, 3.13)	0.077
5 days	-0.44	(-0.96, 0.07)	0.093
4 days	**0.29**	**(0.03, 0.54)**	**0.026***
3 days	**0.13**	**(0.03, 0.22)**	**0.008****
2 days	**0.07**	**(0.02, 0.12)**	**0.004****
1 day	**0.23**	**(0.01, 0.44)**	**0.037***
0 day	-0.01	(-0.08, 0.06)	0.833

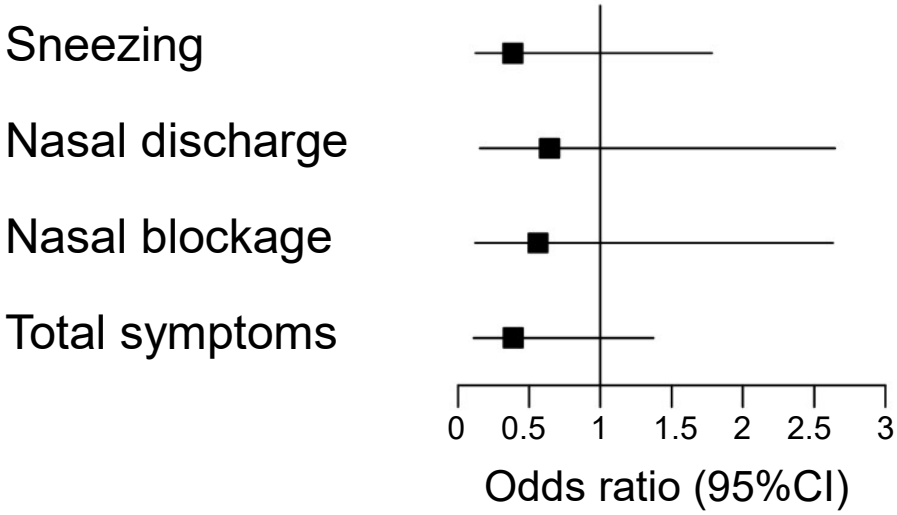
Correlations between Pb levels in ELF (μg/L) and Japanese cedar pollen counts (/cm^2) for 0-9 days before visiting a hospital for sampling are presented. Results presented as regression coefficients (β) and 95% confidence intervals (CIs) with their associated *P* values were obtained by using generalized linear mixed-effects models. The models were adjusted for age, sex, BMI and smoking. Bold values show significant correlations between pollen counts for 1-4 days before visiting a hospital and Pb levels in ELF. $^{*}P < 0.05$, $^{**}P < 0.01$.



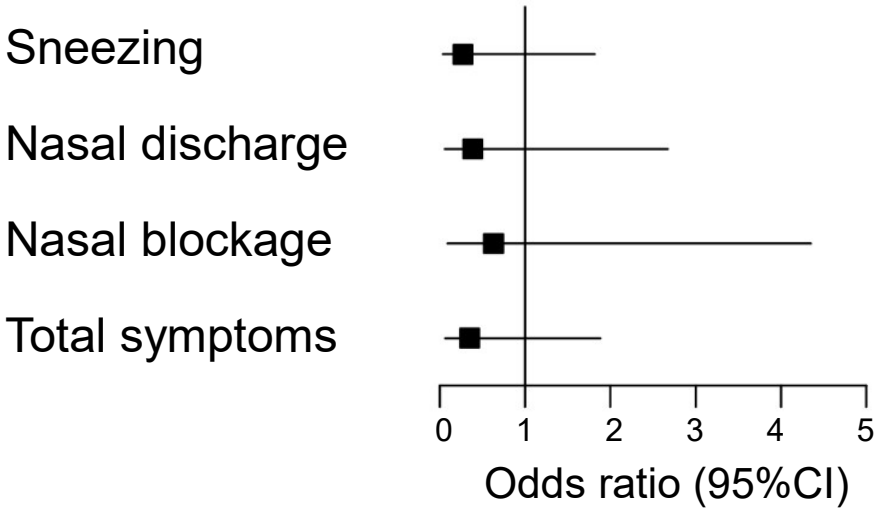
(A) **Pb in ELF**

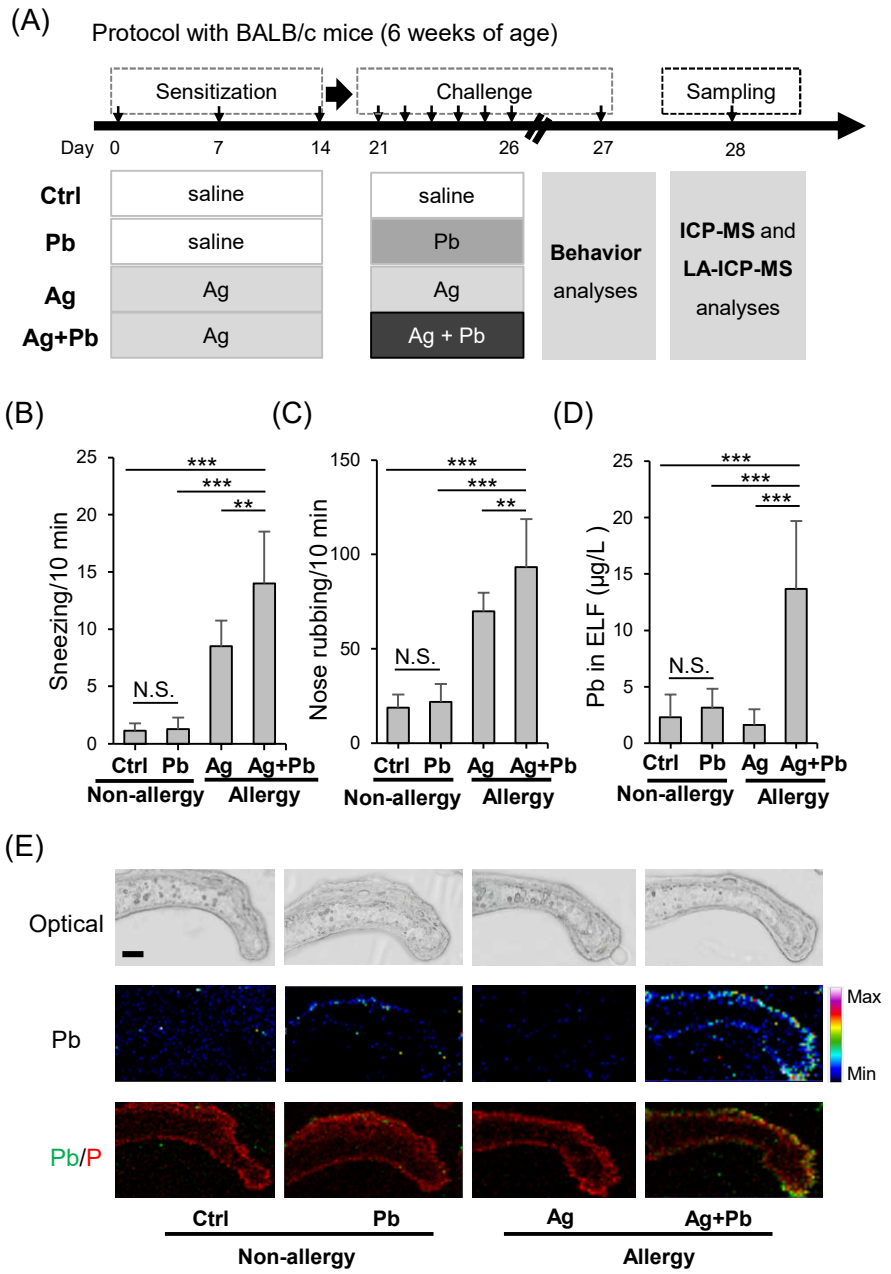


(B) **Pb in saliva**



(C) **Pb in urine**





Supplementary FIGURE LEGENDS

Supplemental Figure S1. Concentrations of Pb, Cd and Hg in Japanese cedar pollen. Absolute values (means ± SD, n=3) of Pb, Cd and Hg measured by inductively coupled plasma mass spectrometry (ICP-MS) in three kinds of Japanese cedar pollen collected in three different areas in Japan are presented.

Supplemental Figure S2. Pollen counts in pre-season and season. Japanese cedar pollen count (/cm^2) in pre-season and that in season are presented. Two-way arrows indicate the periods of pre-season and season.

Supplemental Figure S3. Scores for subjective symptoms in controls and patients in season. Scores for sneezing (A), nasal discharge (B), nasal blockage (C) and total subject symptoms (D) were compared between controls (n = 57) and patients (n=44). The *P* value (***, $P < 0.001$) was calculated by the Mann-Whitney U test.

Supplemental Figure S4. Distribution of Pb in nasal mucosa in the condition after washing. As shown in Figure 3A, mice were intranasally challenged with saline (Ctrl), Pb, Ag or Ag plus Pb (Ag+Pb) after they had been sensitized with an intraperitoneal injection of an antigen (Ag) or a solvent of the Ag (saline). Distribution of Pb in nasal mucosa of nasoturbinate in the condition after washing in each group (n=3-4) is presented. Limited Pb signals on the surface of and inside nasal mucosa were obtained in all groups. Scale bar, 100 μm.

Supplemental Figure S5. Results of histopathological analysis of nasal tissues from mice with and those without allergic rhinitis. Representative photos of hematoxylin & eosin (HE) staining (A, top panels) and Alcian blue-periodic acid-Schiff (PAS) staining (A, middle and bottom panels) are presented. Relative numbers (means ± SD) of goblet cells (B, n=6) and mucins-secreting goblet cells (C, n=4) in non-allergic mice intranasally treated with Pb (closed bar) and allergic mice intranasally treated with an antigen (Ag) and/or Pb (closed bar) to those in control (Ctrl) non-allergic mice without Pb treatment (open bar) are presented. Significant differences were analyzed by ANOVA followed by the LSD test (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$). Scale bar, 50 μm.

Table S1. Median (IQR) concentrations of Cd and Hg in human samples in pre-season and season.

Sample		Pre-season			Season		
		Control (n=57)	Patients (n=44)	*P* - Value[a]	Control (n=57)	Patients (n=44)	*P*- Value[a]
Cd[b]	ELF (μg/L)	ND	ND	-	ND	ND	-
	Saliva (μg/L)	ND	ND	-	ND	ND	-
	Urine (μg/g creatine)	62.4 (41.2, 95.5)	66.6 (42.99, 101.4)	0.995	72.5 (38.8, 127.0)	66.6 (51.3, 103.4)	0.886
Hg[c]	ELF (μg/L)	ND	ND	-	ND	ND	-
	Saliva (μg/L)	ND	ND	-	ND	ND	-
	Urine (μg/g creatine)	ND	ND	-	ND	ND	-

[a]*P* value was calculated by the Mann-Whitney *U* test.

[b]Cd levels in 46.1% of ELF samples and 85.6% of saliva samples were undetectably low (ND). [c]Hg levels in 92.1% of ELF samples, 63.9% of saliva samples and 40.1% of urine samples were undetectably low (ND).

Table S2. Spearman's correlations between scores of each nasal symptom and Pb levels during season

Sample	Sneezing		Nasal discharge		Nasal blockage		Total nasal symptoms	
	r	*P*-Value	r	*P*-Value	r	*P*-Value	r	*P*-Value
ELF	**0.22**	**0.026***	0.12	0.252	**0.34**	**0.001****	**0.25**	**0.013***
Saliva	-0.12	0.238	-0.07	0.517	-0.05	0.630	-0.09	0.384
Urine	-0.17	0.096	-0.13	0.205	-0.07	0.543	-0.14	0.154

$^{*}P < 0.05$, $^{**}P < 0.01$

Table S3. Correlation between Pb levels in saliva and urine samples and Japanese cedar pollen counts

Sample	Time lag	β	95% CI	*P*-Value
Saliva	9 days	0.001	(-0.009, 0.011)	0.862
	7 days	-0.004	(-0.029, 0.022)	0.770
	5 days	0.072	(-0.031, 0.175)	0.173
	4 days	-0.023	(-0.075, 0.029)	0.388
	3 days	-0.008	(-0.028, 0.001)	0.389
	2 days	-0.164	(-0.546, 0.219)	0.402
	1 day	-0.029	(-0.083, 0.026)	0.304
	0 day	-0.010	(-0.024, 0.003)	0.990
Urine	9 days	-0.278	(-0.636, 0.081)	0.129
	7 days	-0.002	(-0.006, 0.002)	0.616
	5 days	-1.747	(-5.635, 2.141)	0.379
	4 days	-0.876	(-2.796, 1.044)	0.371
	3 days	0.118	(-0.610, 0.847)	0.750
	2 days	-0.164	(-0.546, 0.219)	0.402
	1 day	-0.174	(-1.818, 1.470)	0.835
	0 day	0.252	(-0.256, 0.760)	0.332

Correlations between Pb levels in saliva (μg/L) and urine (μg/g creatinine) and Japanese cedar pollen counts (/cm^2) for 9 days before visiting a hospital for sampling are presented. Results presented as regression coefficients (β) and 95% confidence intervals (CIs) with their associated *P* values were obtained by using generalized linear mixed-effects models. The models were adjusted for age, sex, BMI and smoking.

Table S4. Concentrations of Pb in pollens collected in various countries

Sample	Country	Mean (μg/kg)	Range (μg/kg)	References
Japanese cedar pollen	Japan	375	51.9-971.4	This study
Silver birch pollen	Sweden	386	-	This study
Silver birch Pollen	France	2,278	0-5,200	Bellanger et al., 2012[E1]
Orchard grass pollen	Sweden	145	-	This study
Ragweed pollen	China	143	-	This study
Bee pollen	France	240	4-798	Lambert et al., 2012[E2]
Bee pollen	Poland	910	210-3,900	Roman, 2007[E3]
Bee pollen	Romania	-	80-2,080	Harmanescu et al., 2007[E4]

[E1]Bellanger AP, Bosch-Cano F, Millon L, Ruffaldi P, Franchi M, Bernard N. Reactions of airway epithelial cells to birch pollen grains previously exposed to in situ atmospheric Pb concentrations: A preliminary assay of allergenicity. Biol Trace Elem Res. 2012;150:391–5.

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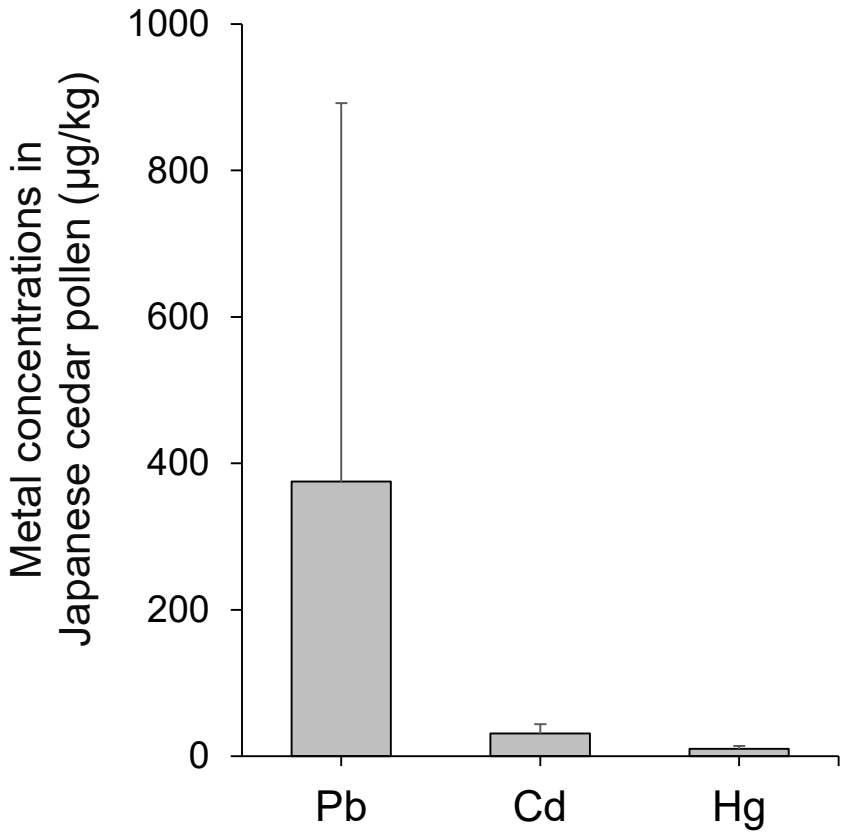
[E3]Roman A. Content of some trace elements in fresh honeybee pollen. Polish J Food Nutr Sci. 2007;57:475–8.

[E4]Harmanescu M, Bordean D, Gergen I. Heavy metals content of bee's pollen from different locations of Romania. Lucr Stiint Med Vet. 2007;40:253–60.



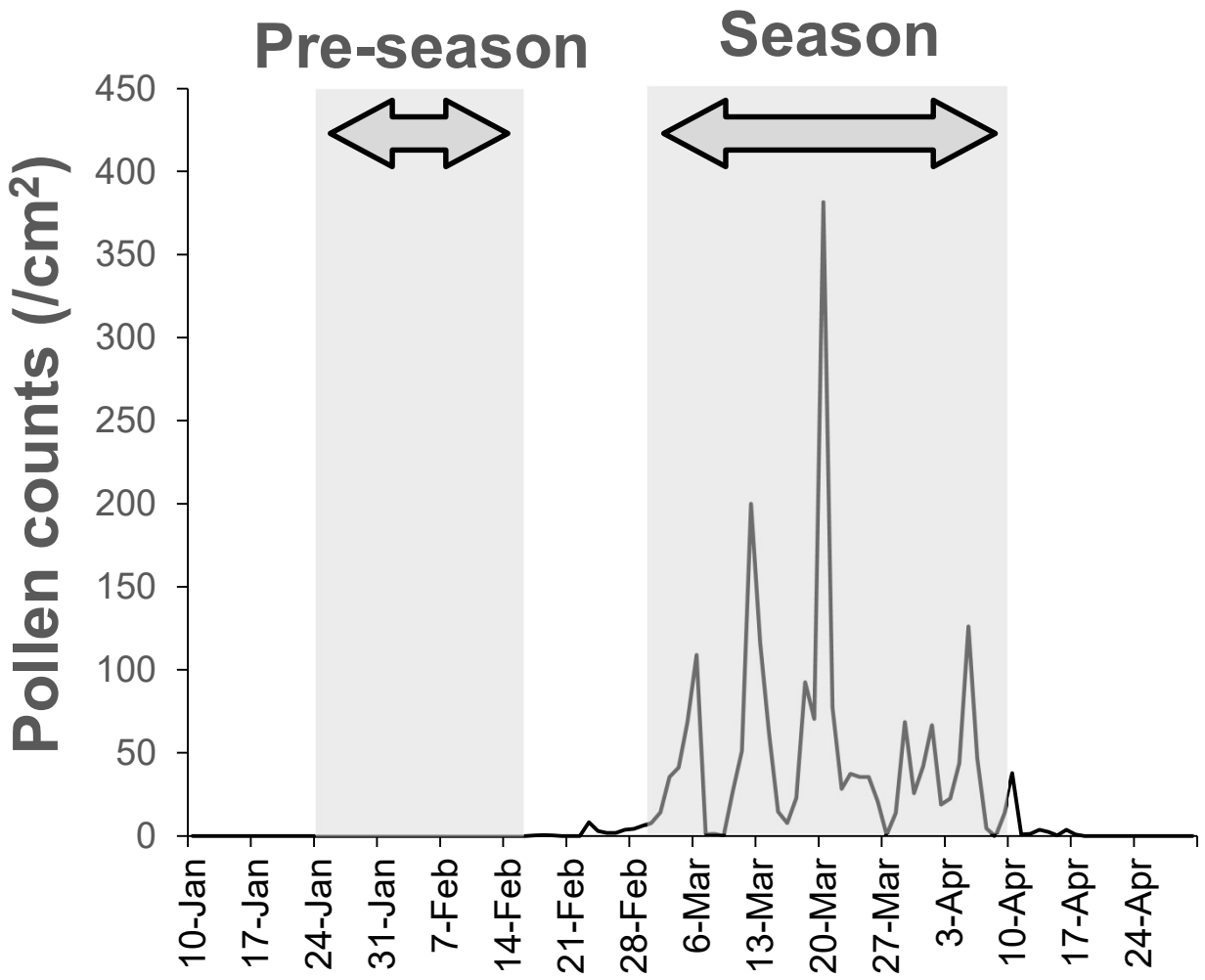


Figure S3

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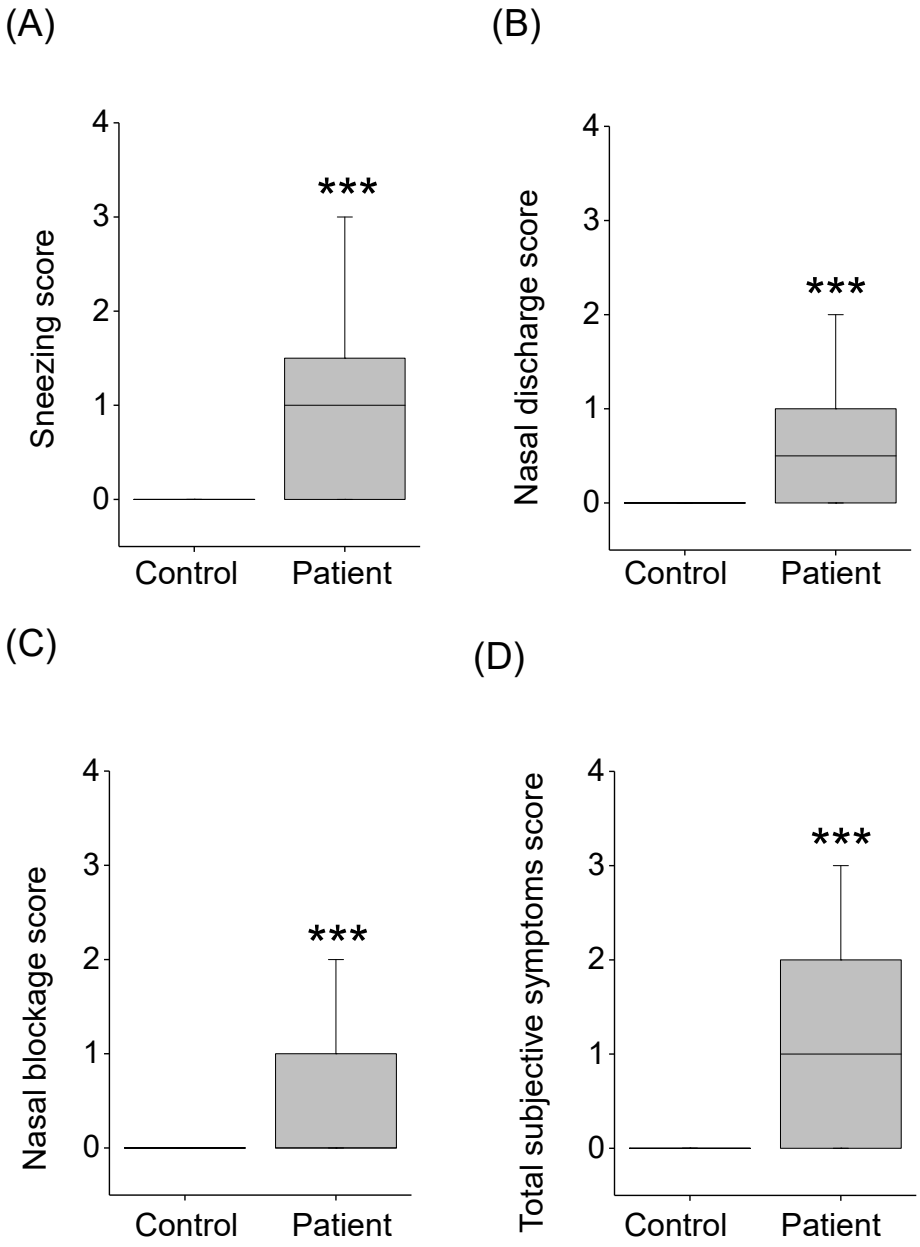

Figure S4

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