Int J Clin Exp Med 2019;12(7):8807-8814
www.ijcem.com /ISSN:1940-5901/IJCEM0088495

Original Article

Effects of atmospheric pollutants on risks of *mycoplasma pneumoniae* infections in outpatients during warm and cold seasons in China

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Received November 16, 2018; Accepted June 5, 2019; Epub July 15, 2019; Published July 30, 2019

Abstract: The aim of the current study was to investigate the impact of 3 atmospheric pollutants, $PM_{2.5}$, SO_2, and O_3-8h, on *M. pneumoniae* infection risks during warm and cold seasons in Shengyang, China. The relationship between daily average concentrations of $PM_{2.5}$, SO_2, and O_3-8h, between April 1, 2013, and March 31, 2016, and *M. pneumoniae*-positive patients in the Department of Pediatrics and Respiratory Clinic were analyzed via Spearman's correlation analysis. A time-series Poisson regression generalized additive model (GLM) was used to analyze risks for testing positive for *M. pneumoniae*, associated with differences in $PM_{2.5}$, SO_2, and O_3-8h concentrations during warm and cold seasons (1). Daily average concentrations of these 3 atmospheric pollutants were related to the number of patients testing positive for *M. pneumoniae* ($P<0.05$). The relationship was stronger for females than males. The relationship was stronger for patients with positive results ($P<0.01$) than patients with negative results ($P<0.05$). Pediatric outpatients (under 15 years of age) showed a stronger relationship to $PM_{2.5}$ ($P<0.01$). Patients over 15 years of age (respiratory clinics) showed a stronger relationship to SO_2 and O_3-8h ($P<0.01$) (2). Average daily concentrations of $PM_{2.5}$, SO_2, and O_3-8h exhibited different characteristics. Increased risks for *M. pneumoniae* infections were related not only to mass concentrations of atmospheric pollutants, but also to physiological doses of atmospheric pollutant particles.

Keywords: M. pneumoniae, atmospheric pollutants, risk

Introduction

Mycoplasma pneumoniae infections are main causes of community-acquired pneumonia in children [1-4]. A worldwide pathogen survey of community acquired pneumonia (CAP) in adults showed that *M. pneumoniae* accounts for 12% of CAP [5]. However, proportions of *M. pneumoniae* in CAP were as high as 26.7% and 22.3% in Shanghai and Beijing, respectively. *M. pneumoniae* has surpassed *Streptococcus pneumoniae*, becoming the primary pathogen of CAP in adults.

Recent studies have reported that peak worldwide prevalence of *M. pneumoniae* infection often occurs in Fall and Winter [6, 7]. Atmospheric concentrations of pollutants may exceed standards and environmental pollution is often the heaviest during these seasons. This phenomenon has received much attention. Onset of clinical lesions caused by *M. pneumoniae* (MP), as well as mechanisms underlying these lesions, ranging from self-limiting to lethal, intra-lung to extra-lung, and limited divergence to outbreaks, are not fully understood [8-15].

The current study analyzed the relationship between $PM_{2.5}$, SO_2, and O_3-8h concentrations and the number of MP-positive outpatients. Moreover, this study evaluated the effects of $PM_{2.5}$, SO_2, and O_3-8h concentrations on risks of MP infections during warm and cold seasons in China.

Materials and methods

Subjects

The current study sampled 28,155 outpatients in the Department of Internal Medicine and Pediatrics of the Affiliated Central Hospital of

Shenyang Medical College, between April 1, 2013, and March 31, 2016. Patients with a history of asthma, chronic coughing, and repeated respiratory infections were excluded. Patients with anemia, low immunity, congenital diseases, such as congenital heart disease and congenital hypothyroidism, and genetic or metabolic diseases, were also excluded. Total patients included 13,651 males and 14,504 females, aged 1 to 99 years, with an average age of 25.8±27.9 years. This study was conducted in accordance with the Declaration of Helsinki. It was conducted with approval from the Ethics Committee of Shenyang Medical College. Written informed consent was obtained from all participants.

MP detection

SERODIA-MYCOII reagent, used as a gelatin particle agglutination test (PA) reagent for detection of mixed antibodies of IgM and IgG, was obtained from Fujirui Co., Ltd., Japan. All operations were carried out according to manufacturer instructions. Specific methods: Proportionately-diluted serum samples and negative/positive control substances were added to a U-shaped reaction plate (25 μl per well). Unsensitized and sensitized artificial gelatin particles (25 μl per well, respectively) were added, while mixing and shaking. The mixture was allowed to stand at room temperature (15~30°C) for 3 hours. Results were then read on a plate observer. Dilution was ≥ 1:40 (serum to positive).

Sample locations and sampling times of $PM_{2.5}$

The sampling point was located on the Fifth floor of Shenyang Environmental Monitoring Center Station, Shenhe District, Shenyang. This location is 15 m above ground with no surrounding obstructions. A secondary level road, which has large traffic flow, ran 50 m to the northeast of the sampling point. There are no large factories around this area. It is a commercial residential area with no artificial pollution sources. After sample collection, the dust film was immersed in ultrapure water for 20 minutes. It was subjected to ultrasonic elution (ultrasound, SIUICTS-35A, China) and centrifuged for 20 minutes at 14,000 r/min. When the samples became numerous, they were repeatedly centrifuged for collection (D-37520 Osterode, Thermo fisher, Germany), then freeze-dried (GT2-90, SKR Systems UG, Germany). Residue was weighed, dust content was calculated, and the sample was preserved at -20°C. For analysis of sample elements, approximately 0.05 g of the sample was placed in a Teflon beaker. It was treated, in turn, with 5 mL of hydrofluoric acid, 10 mL of nitric acid, and 1 mL of perchloric acid. It was then heated to 220°C. A large amount of white smoke was emitted. Next, the sample was heated slowly until dry. After cooling, 1 mL of hydrochloric acid and 10 mL of water were added. The mixture was boiled on a hot plate at 220°C for 5 minutes. It was then cooled and made up to 20 mL with water in a colorimetric tube for relevant testing. *Tests*: Pb atomic absorption spectrophotometry (AAS); Other elements included inductively-coupled plasma optical emission spectrometry (ICP-AES) (Atomic Absorption Spectrometer AA-7003, Beijing Dongxi Electronics, Inductively Coupled Plasma Emission Spectrometer SPS8000, Seiko, Japan).

Statistical analysis

The frequency module in description statistics was used to derive the number of daily and monthly visits from outpatient medical records. The module was also used to establish a system, including patient numbers with different valence ratios, as well as fusion of $PM_{2.5}$ data with the number of visits. Spearman's rank correlation analysis was used to analyze the relationship between the number of patients (based on gender, age, season, warm/cold season) and atmospheric pollutants. Positive and negative typing of the *M. pneumoniae* antibody was used to describe the utility rate index. Intergroup comparisons were performed using Chi-square tests. Statistical significance is set at $P<0.05$. Stata19.0 was used for analysis. Compared to the general population, the daily outpatient number and the number of MP-positive patients are small probability events. Therefore, regarding time series data, the actual distribution approximated the Poisson distribution. The time-series Poisson regression generalized additive model (GLM) was used. The specific model was:

$$\text{Log}[E(Y_i)] = \alpha + \sum_{i=0} B_i X_i + \sum_{j=0} f_j Z_j$$

Yi: Observation day; i: Number of people tested on the day, E (Yi): Expected value of the number of people tested on observation day i; α: Intercept; X: Indicator variable of linear variable

Table 1. Situation of atmospheric fine particle concentrations and number of MP detection in the heating season and non-heating season

Atmospheric particle concentration	Heating season		Non-heating season		*P*
	N	$\bar{x}\pm s$	N	$\bar{x}\pm s$	
$PM_{2.5}$ (μg/m^3)	453	92.23±67.18	642	54.70±39.58	<0.001
SO_2 (μg/m^3)	453	146.97±71.56	642	34.28±20.45	<0.001
O_3 (μg/m^3)	453	45.19±24.78	642	97.04±41.03	<0.001
Number of MP detection (n/d)	20705	45.61±15.84	21998	34.26±10.44	<0.001
Negative	10550	23.24±8.96	12919	20.12±6.94	<0.001
Positive	10155	22.37±10.91	9079	14.14±6.61	<0.001

Note: O_3: Maximum 8-hour sliding average (O_3-8 h); The heating season was 5 months (starting on November 1st and ending on March 31 in the next year); The non-heating season was 7 months (starting on April 1st and ending on October 30th).

of the corresponding variable; β: Estimated indicator variable coefficient by the regression model: Z: Indicator variable of non-linear variable of the corresponding variable, Σ: sum.

Through single factor fitting of GLM and bias testing, the impact of atmospheric pollutants on the number of outpatients and the number of MP testers was analyzed. Relative risk (RR) was used to evaluate hazard sizes.

Considering possible hysteresis effects of pollutants on the number of detections, Lag-day (Lag) was selected from 1 to 7 days, analyzing the impact of atmospheric pollutants on the number of people tested.

Results

During warm and cold seasons, daily average concentrations of the three atmospheric pollutants, outpatient numbers in the Department of Pediatrics and Respiratory Medicine, and the numbers of MP-positive patients were monitored.

During the test period, a total of 28,155 patients visited the Department of Respiratory Disease (15 years old and above) and Department of Pediatric Outpatients (15 years old and younger). The mean daily outpatient number in the Department of Respiratory Disease was 335.1±106.5. Of the mean daily outpatients, 251.0±81.5 included 13,651 males and 14,504 females. They were aged 1 to 99 years, with an average of 25.8±27.9 years. Atmospheric fine particulate matter for the warm season ($PM_{2.5}$: 92.23±67.18 μg/m^3; SO_2: 146.97±71.56 μg/m^3; and O_3-8h: 45.19 ±24.78 μg/m^3) was significantly different, compared to atmospheric fine particulate matter for the cold season ($PM_{2.5}$: 54.70± 39.58 μg/m^3; SO_2: 34.28±20.45 μg/m^3; and O_3-8h: 97.04±41.03 μg/m^3); (P<0.001). $PM_{2.5}$ and SO_2 concentrations in the warm season exceeded national ambient air quality standards (GB 3095-2012). The number of MP detections during the same period was significantly different from that in the cold season (**Table 1**).

During the same period, composition analysis of $PM_{2.5}$ samples indicated that $PM_{2.5}$ was composed of sulfate, nitrate, organic carbon (high content), and various elements (Al, Ti, As, Ba, Cu, Cr, Fe, Cd, Se, Ni, Mn, Zn, or Pb). Al and Ti contents were much higher than the other elements. Compositions were different in different seasons. Warm-season $PM_{2.5}$ contained organic carbons (43.7%), elemental carbon (17.4%), heavy metals (0.727%), water-soluble cations (9.82%), and anions (23.17%). Cold-season $PM_{2.5}$ contained organic carbon (27.1%), elemental carbon (11.34%), heavy metals (0.540%), water-soluble cation (6.34%), and anions (16.48%).

Relationship between daily average concentrations of atmospheric pollutants (all year) and numbers of MP detections

All-year daily average concentrations of $PM_{2.5}$, SO_2, and O_3-8h were related to the number of MP detections (P<0.05). Of these, $PM_{2.5}$ and SO_2 were positively correlated with the number of MP detections, while O_3-8h was negatively correlated. The relationship to gender was statistically significant (P<0.01). Females were

Table 2. Spearman's rank correlation analysis between atmospheric pollutant concentrations and number of MP detection in outpatients

Pollutant ($\mu g/m^3$)	Patient tested	Gender		Age		MP test result	
		M	F	15 years old or younger	15 years old or older	Negative	Positive
$PM_{2.5}$	0.262** (5)	0.241** (3)	0.398** (4)	0.352** (5)	0.183** (5)	0.253** (5)	0.136** (4)
SO_2	0.366** (2)	0.303** (1)	0.252** (2)	0.212** (2)	0.345** (4)	0.364** (2)	0.229** (3)
O_3	-0.219** (5)	-0.173** (1)	-0.230** (3)	-0.154** (7)	-0.200** (4)	-0.258** (0)	-0.079* (3)

Note: The most significant Lags were marked in parentheses, **P<0.01, *P<0.05. O_3: maximum 8-hour sliding average (O_3-8h).

more sensitive to atmospheric pollutants than males. Concerning the relationship with the number of MP detections, test-positive patients (P<0.01) showed a stronger relationship than test-negative patients (P<0.05). The relationship to age structure (pediatric outpatient number and respiratory medicine outpatient number) was also statistically significant (P<0.01). Patients under 15 years old (pediatric clinics) showed a stronger relationship with $PM_{2.5}$, while those over 15 years old (respiratory clinics) showed a stronger relationship to SO_2 and O_3-8h.

The lag period of the 3 pollutants was within 1-7 days, concentrated mainly within 2-5 days. Hysteresis effects indicated that MP infections effected by pollutants may occur within a week, followed by an increase in the number of outpatient visits to Pediatric and Respiratory Medicine Clinics (**Table 2**).

Correlation analysis and hysteresis effects of three atmospheric fine particle concentrations and positive MP antibody numbers

Single factor GLM was used to analyze the impact of $PM_{2.5}$, SO_2, and O_3-8h concentrations on the presence of MP positive antibodies during warm and cold seasons (**Table 3**). Analysis showed that the daily average $PM_{2.5}$ concentrations during the warm season were positively correlated with the number of MP-positive antibodies. However, a lag of 0-7 days for $PM_{2.5}$ showed no statistically significant association with the number of MP-positive antibodies or hysteresis effects (**Figure 1**). There was a positive correlation between SO_2 and the number of MP-positive antibodies. Lag2 concentrations showed the most significant impact on the number of MP positive antibodies (P<0.001). RR values and 95% CIs were increased by 1.0% [95% CI (0.7-1.2) %], respectively, for every SO_2 concentration increase of 10 $\mu g/m^3$ (**Figure 2**). O_3-8h was negatively correlated with the number of MP-positive antibodies. It was the highest on the day it was observed, showing significant effects on Lag 0-7 (P<0.001) (**Figure 3**). During the cold season, $PM_{2.5}$ was positively correlated with the number of MP-positive antibodies. Its concentration had the most significant effects on Lag 7 (P<0.001). For every 10 $\mu g/m^3$ increase of $PM_{2.5}$ concentrations, RR values and 95% CIs increased by 2.4% [95% CI (1.8-2.4) %], respectively. SO_2 was also positively correlated with the number of MP-positive antibodies, with the most significant impact on Lag 5 (P<0.001). For every 10 $\mu g/m^3$ increase of SO_2 concentrations, RR values and 95% CIs increased by 5.7% [95 % CI (4.4-7.1) %], respectively. Only the O_3-8h concentration on Lag 2 showed a positive correlation with the number of MP-positive antibodies. This was statistically significant (P<0.05). For every 10 $\mu g/m^3$ increase of O_3-8h concentrations, RR values and 95% CIs increased by 0.7% [95% CI (0.2-1.2) %], respectively.

Discussion

The current study investigated the relationship between 3 atmospheric pollutants ($PM_{2.5}$, SO_2, and O_3-8h) and the number of MP-positive outpatients in the Department of Respiratory Clinics and Pediatrics in Shenyang during warm and cold seasons, between April 1, 2013 and March 31, 2016. Results showed that the 3 atmospheric pollutant concentrations and the numbers of MP-positive patients showed significant differences between warm and cold seasons. $PM_{2.5}$ and SO_2 concentrations exceeded national ambient air quality standards (GB

Table 3. Relationship of concentrations of $PM_{2.5}$, SO_2, and O_3 with number of positive MP antibody in different seasons in 2013.4.1-2016.3.31

Variable	Lag	Heating season		Non-heating season	
		RR (95% CI)	*P*	*RR (95% CI)*	*P*
$PM_{2.5}$ 10 μg/m³	Lag0	1.000 (0.999, 1.002)	0.001**	1.006 (1.002, 1.011)	0.010
	Lag1	1.001 (0.999, 1.004)	0.315	1.010 (1.004, 1.016)	0.001**
	Lag2	1.000 (0.998, 1.003)	0.738	1.010 (1.004, 1.016)	0.001**
	Lag3	1.001 (0.998, 1.004)	0.458	1.016 (1.010, 1.022)	0.000**
	Lag4	1.002 (0.999, 1.004)	0.204	1.019 (1.013, 1.025)	0.000**
	Lag5	1.000 (0.998, 1.003)	0.753	1.020 (1.014, 1.026)	0.000**
	Lag6	1.001 (0.999, 1.004)	0.254	1.018 (1.011, 1.024)	0.000**
	Lag7	1.000 (0.998, 1.002)	0.891	1.024 (1.018, 1.030)	0.000**
SO_2 10 μg/m³	Lag0	1.009 (1.006, 1.011)	0.000**	1.038 (1.027, 1.049)	0.000**
	Lag1	1.009 (1.007, 1.012)	0.000**	1.047 (1.035, 1.060)	0.000**
	Lag2	1.010 (1.007, 1.012)	0.000**	1.047 (1.034, 1.059)	0.000**
	Lag3	1.008 (1.005, 1.010)	0.000**	1.051 (1.038, 1.064)	0.000**
	Lag4	1.007 (1.005, 1.010)	0.000**	1.056 (1.042, 1.069)	0.000**
	Lag5	1.008 (1.005, 1.010)	0.000**	1.057 (1.044, 1.071)	0.000**
	Lag6	1.008 (1.005, 1.010)	0.000**	1.055 (1.042, 1.068)	0.000**
	Lag7	1.007 (1.004, 1.009)	0.000**	1.052 (1.039, 1.065)	0.000**
O_3 10 μg/m³	Lag0	0.957 (0.948, 0.965)	0.000**	1.002 (0.997, 1.008)	0.335
	Lag1	0.959 (0.950, 0.967)	0.000**	1.002 (0.997, 1.007)	0.442
	Lag2	0.964 (0.956, 0.973)	0.000**	1.007 (1.002, 1.012)	0.009**
	Lag3	0.964 (0.955, 0.972)	0.000**	1.005 (1.000, 1.010)	0.052
	Lag4	0.968 (0.959, 0.976)	0.000**	1.002 (0.997, 1.007)	0.402
	Lag5	0.962 (0.953, 0.970)	0.000**	1.000 (0.995, 1.006)	0.850
	Lag6	0.965 (0.957, 0.974)	0.000**	0.995 (0.990, 1.000)	0.053
	Lag7	0.968 (0.959, 0.976)	0.000**	0.995 (0.989, 1.000)	0.041*

Note: OR: relative risk, 95% CI: 95% CI, Lag0 reflects the death due to circulatory system diseases on the day, Lag1, 2, 3, 4, 5, 6, and 7 reflect the number of positive MP antibody on Lag 1, 2, 3, 4, 5, 6, and 7. $PM_{2.5}$: fine particulate matter, SO_2: sulfur dioxide, O_3: ozone; *P<0.05, **P<0.01.

3095-2012) during the warm season, indicating that an increase in the concentrations of air pollutants during the warm season (winter) was directly related to negative effects on health.

M. pneumoniae (MP) was first isolated and cultured by Nocard and Roux in 1898 [16]. MP, which belongs to the genus *Mycoplasma*, can survive *in vitro* without relying on living cells. One of the smallest microorganisms that can self-copy, it is taxonomically placed between viruses and bacteria. It has no cell wall. It is a conditional pathogenic microorganism widely found in nature. Its inherent biological characteristics determine the ease with which it has become a $PM_{2.5}$ carrier component, invading the respiratory tract and positioning itself in the crypt of cilia via a sliding motion. It adheres to the receptor on the surface of epithelial cells with its tip-specific structure, resisting clearance by mucosal cilia and phagocytosis by phagocytic cells. In addition, it may produce a range of cytotoxic effects.

At present, serological testing is the main method used for diagnosis of MP in laboratory settings [17]. Under clinical conditions, the gelatin particle agglutination method has been widely used due to its sensitivity, simple operation, rapid results, and low cost. The current study analyzed changes in the number of MP antibodies in Pediatric and Respiratory Medicine clinics. In addition to temperature-related seasonal factors, atmospheric pollutants may be important contributors to peak MP infections seen in recent years [18]. The findings of this study indicate that higher daily average concentrations of $PM_{2.5}$ and SO_2 are related to greater

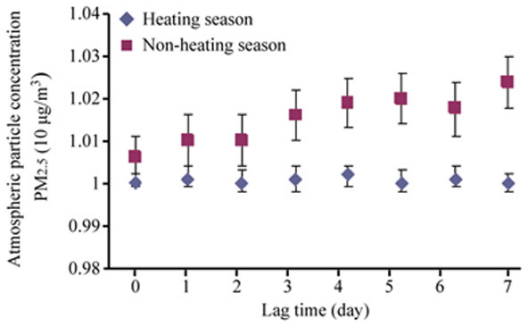

Figure 1. Relationship of concentrations of PM2.5 with number of positive MP antibody in different seasons.

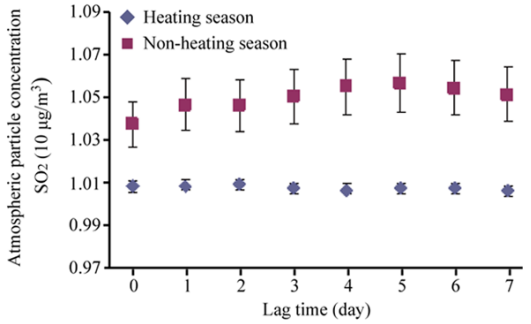

Figure 2. Relationship of concentrations of SO_2 with number of positive MP antibody in different seasons

numbers of MP-positive cases. The effects of atmospheric pollutants also varied with gender. Results showed that adults over the age of 15 years were more sensitive to SO_2 and O_3-8h, while females and children under 15 years (pediatric clinics) were more sensitive to fine particulate $PM_{2.5}$. It is evident that $PM_{2.5}$ exerts a greater negative effect on women and children. There may be several causes for this, as follows: 1) It is related to the characteristics of $PM_{2.5}$, where atmospheric suspended fine particles (PM), which are ≤ 2.5 m in diameter and have strong adsorption capacity, act as carriers and catalysts of a variety of inorganic, organic, and biological contaminants. This is compounded by the fact that metals carried by $PM_{2.5}$ may have synergistic effects with *M. pneumoniae*, inducing oxidative stress. This then regulates the host immune system and inflammatory response via TLRs and/r NF-kB pathways [19]; 2) It is associated with the physiological characteristics of children in the growth and development stage. In this stage, various immune cells in the normal immune response

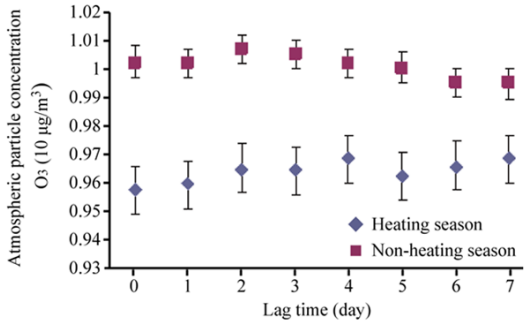

Figure 3. Relationship of concentrations of O_3 with number of positive MP antibody in different seasons.

processes do not cooperate. Thus, their ability to remove antigenic foreign bodies is weak. In addition, the respiratory system is relatively sensitive and susceptible to respiratory symptoms. Once infected, most children are taken care of by their mothers (female). Close contact increases the potential for infections, accelerating the symptoms of respiratory infections on re-exposure to atmospheric pollutants [20]. These are postulated as the causes for increased numbers of children and women attending clinics during smoggy periods.

In this study, single factor GLM was used to analyze the risk of positive MP detections associated with $PM_{2.5}$, SO_2, and O_3-8h concentrations during warm and cold seasons. Present results showed that higher concentrations of $PM_{2.5}$ and SO_2, during the warm season, were associated with higher numbers of patients with MP-positive antibodies. $PM_{2.5}$ had no hysteresis effects. SO_2 on Lag2 had the strongest effects on patient numbers with MP-positive antibody detection. For every 10 μg/m^3 increase in SO_2 concentrations, the risk of detecting a patient with MP-positive antibodies was increased by 1.0%. During the cold season, $PM_{2.5}$ and SO_2 concentrations were positively correlated with the number of patients with MP-positive antibodies. $PM_{2.5}$ on Lag7 showed the strongest effects. The numbers of patients with MP-positive antibody detection were increased by 2.4% for every 10 μg/m^3 increase in $PM_{2.5}$ concentrations. SO_2 on Lag5 showed the strongest effects. The number of patients with MP-positive antibodies increased by 5.7% for every 10 μg/m^3 increase in SO_2 concentrations. It is evident that $PM_{2.5}$ and SO_2 contribute to increased numbers of patients

with MP-positive antibodies during both warm and cold seasons. However, in the cold season, both increased the risk of MP-positive antibody detection. This suggests that increased risks for patients with MP-positive antibody detection is not only related to mass concentrations of atmospheric pollutants but is also determined by relevant physiologic doses of atmospheric pollutant particles.

In summary, air pollutants are closely related to increases in the number of patients with MP detection, especially in children and women. $PM_{2.5}$ and SO_2 may contribute to increased MP risks during warm and cold seasons. However, the risk is greater during the cold season. Although this study had several limitations, it may provide direction for subsequent research on this topic.

Acknowledgements

The current study was funded by the Shenyang Science and Technology Plan Project (F16-156-9-00) and Science and Technology Research Project of Liaoning Provincial Department of Education (L2014419).

Disclosure of conflict of interest

None.

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