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Home and day-care microenvironment exposure to *Blomia tropicalis* allergens and their associations with salivary eosinophilic cationic protein (ECP) among preschool children in Singapore

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Abstract

To date, exposure studies linking dust-mite allergens with asthma and allergic morbidities have typically relied on sampling from representative locations in the home for exposure assessment. We determine the effects of differing microenvironments allergen exposures on asthma and asthma severity among 25 case and 31 control preschool children in Singapore. Blo t 5 allergen levels in various niches from the children's home and day-care microenvironments as well as their Blo t 5 time-weighted concentrations were determined. Eosinophilic cationic protein (ECP) levels from the children's saliva as markers for airway inflammation were obtained. Salivary ECP levels were higher in children with asthma than those without and the strength of association increased with higher salivary ECP levels. Although there was no relationship between time-weighted Blo t 5 concentrations with salivary ECP levels among the controls, a positive statistically significant relationship was noted among cases, demonstrating the effects of cumulative exposure on asthma severity. Avoidance measures to reduce Blo t 5 allergen exposure should include all microenvironments that asthmatic children are exposed throughout the day.

KEYWORDS

allergen, microenvironment exposure, preschool children, salivary ECP, tropics

1 | INTRODUCTION

The validity and precision of research on asthma risk from dust-mite allergens depend on the quality of exposure information.¹ Previous studies have been conducted to measure dust-mite allergen exposures that were obtained only from the home environments where children spend most of their time.^{2,3} In most studies, settled-dust sampling was only performed from home dust reservoirs such as floors and mattresses.^{1,4} However, it is not clear which sampling locations best represents exposures for a child living through different

microenvironments. Although Zhang et al⁵ showed that homes had significantly higher concentrations and prevalence of allergens compared with the other locations, research evidence has reported that children can be exposed to clinically important levels of indoor allergens in locations outside their home, such as day-care centers.⁶⁻⁸ Additionally, the cumulative effects of allergen exposures from different microenvironments are unclear. Indeed, in many parts of the world, children can spend up to 10 hours a day away from the home microenvironment in day-care centers.^{8,9}

The risk-relevant exposure parameter should, in theory, represent the individual child's breathing zone concentration of the

allergen over a time period that is relevant to the risk of developing an asthma outcome.¹ Thus, measurements conducted in a single location at home are at best exposure surrogates that correlate to a greater or lesser extent with the risk-relevant exposure parameter.¹ Unfortunately, no studies have been done on asthma-related outcomes resulting from exposure assessments conducted in multiple microenvironments simultaneously. This will leave possible confounding between exposure to one microenvironment and the other unaddressed. Indeed, a major knowledge gap is the lack of simultaneous measurements in the home as well as day-care microenvironments that can provide more complete information that addresses this knowledge gap.

To determine the effects of differing microenvironments allergen exposures on asthma among preschool children, we measured allergen levels in various niches of their home and day-care microenvironments and compared them with their asthma status as well as eosinophilic cationic protein (ECP) levels as markers for asthma severity. The association between eosinophilia and asthma was first observed shortly after eosinophils were discovered. In asthmatic patients, eosinophils are present in increased numbers in the blood, sputum, and bronchoalveolar lavage fluid.¹⁰ Upon activation, eosinophils can release granulocyte-derived proteins, the most toxic of which are eosinophil cationic protein (ECP) and major basic protein (MBP).¹¹ ECP can be found in many body fluids (eg, serum and sputum), and it has been established to reflect airway inflammation and asthma severity.¹² Indeed, salivary ECP has been reported to be useful in assessing asthma severity.^{11,13} We measured the Blo t 5 allergen from the *Blomia tropicalis* house dust-mite as it has been reported that it occurs in a significant percentage of homes in Singapore and that the allergenic components from this mite are important triggers of allergies in the tropics.^{5,14} A high prevalence of *B tropicalis* sensitization occurs and is strongly associated with the presentation of allergic diseases in Singapore.¹⁴

2 | MATERIALS AND METHODS

2.1 | Study design

We recruited 56 preschool children from 31 childcare centers in this study. Questionnaire data, dust, and salivary samples were obtained between 2004 and 2006. House dust and salivary samples were collected on the same day. Their information on health, family, and home characteristics were collected using the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaire from the parents of the children as detailed elsewhere.¹⁵ Of these, we classified the children into cases and controls. The selection criteria for the cases were as follows: (a) doctor diagnosed asthma and (b) wheeze symptoms in the past 12 months. Inclusion criterion for the controls was that the children should have no asthma or allergic symptoms in the questionnaire. For both groups, the children should not have rebuilt/renovated their homes because

Practical implications

- Measurements of exposure linking to health outcomes have typically focussed on one microenvironment.
- This study showed that asthma severity among asthmatics is positively linked to the effects of cumulative allergen exposure from various microenvironments.
- Avoidance measures to reduce allergen exposure should include all microenvironments that asthmatic children are exposed throughout the day.

of moisture problems and not have changed residence since the questionnaire. Informed consent was obtained from a parent, and ethics approval was given by the National University of Singapore Institutional Review Board.

2.2 | Allergen exposure assessment

Dust samples were collected using a modified Kirby Classic III (Kirby Co) vacuum cleaner which was adapted with a chamber that collects dust onto a filter paper as detailed elsewhere.⁹ For each child, four to six dust samples were collected from the house as well as day care where they attended. These samples were from the child's day-care classroom floor, day-care mattress, home bedroom floor, and home mattress. For day-care classroom floor, dust was vacuumed in the middle portion (an area of approximately 2 m²) and along the perimeter of the floors (about 2 m²) for 5 minutes. For day-care mattresses, dust was vacuumed from the mattress surface (about 1 m²) for about 1 minute. For home bedroom floor, dust was vacuumed in the child's bedroom floor (an area of approximately 2 m²) and along the perimeter (about 2 m²) for 5 minutes. For home mattress, dust was vacuumed on mattress surface (about 1 m²) for about 1 minute. If a carpet was present in the home and day care, at least 1 minute was devoted to sampling the carpet.

The dust collected were sealed in grip lock bags, stored at 4°C in coolers and transported back to the laboratory and immediately be placed into a freezer at -20°C. In the laboratory, dust samples were sieved through a No. 45 mesh screen 355-μm pore-size grating to remove large particles and fibers. The weight of the recovered dust was then noted. The fine dusts were suspended in phosphate-buffered saline (pH 7.4) in a proportion of 100 mg (±5 mg) fine dust per 2 mL buffer. The dust suspensions (resuspended using a vortex mixer) were mixed end-over-end overnight for 16 hours on a laboratory rocker at 4°C. The supernatants were harvested (in 1 mL) by centrifugation (20 minutes at 1321 g at 4°C) and frozen at -20°C to be assayed. Sandwich ELISA for Blo t 5 was measured. Ninety-six well microtiter plates were coated with monoclonal antibodies at a concentration of 10 μg/well. After washing, the plates were blocked with phosphate-buffered saline with Tween (PBST) for about an hour at room temperature followed by washing and incubation with dust

samples at room temperature for another hour. Standard curves were constructed using the reference standards provided. After extensive washings, incubation with horseradish peroxidase anti-rabbit antibody was performed for 1 hour at room temperature. Assay was developed using 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) for 30 minutes. The plate was read at absorbance of 655 nm. All the dust assaying kits used were purchased from Indoor Biotechnologies Ltd.

We estimated the total exposure of a child to Blo t 5 allergens using the microenvironmental model.^{1,16} In this model, exposure of a child to Blo t 5 allergens was defined as the time-weighted average Blo t 5 concentrations encountered as the child moves through a series of microenvironments. We multiply measurements taken in separate microenvironments by the time spent there divided by the total time according to Equation 1.

$$C_{TW} = \frac{\sum t_{ij} \cdot C_{ij}}{t_{total}} \quad (1)$$

where C_{TW} , time-weighted Blo t 5 concentration for period $i = 1, \dots, 3$, where j is the microenvironment, C_{ij} is the Blo t 5 concentration in microenvironment j in period i , t_{ij} is the number of hours spent in microenvironment j in period i . Concentration of Blo t 5 from the home floors, mattresses, day-care center floors, and day-care mattresses was used. An assumption used in this model is that the Blo t 5 allergen concentration levels at each site are uniform across the study period. The environment in the tropics does not vary significantly to cause seasonal dust-mite allergen levels.⁵ Corresponding time periods for different microenvironments were obtained from the report of child's day-care and home schedules.

2.3 | Salivary ECP assessment

Saliva samples were collected in the morning to minimize circadian variation of salivary biomarkers. We followed Schmekel et al's laboratory method to analyze saliva ECP¹³ and full details on ECP sample collection and analysis have been reported elsewhere.¹⁷ Briefly, we adopted additional precautionary measures to avoid variable attachment of ECP to test-tube walls and mucus. After rinsing the oral cavity thoroughly with tap water, 1 mL of passively drooled saliva was collected via a funnel into a polypropylene tube (Falcon® tubes do not bind ECP). Saliva samples were stored at -70°C till analysis. 40 μL aliquots were diluted 4:1 with 10% sputolysin (a concentrate of dithiothreitol in phosphate buffer) to isolate epithelial cells. Samples were vortexed for 30 seconds and then incubated for 15 minutes with agitation at room temperature. Following this, samples were diluted 2:1 with 0.4% CTAB (N-Cetyl-NNN-trimethylammonium bromide with pH adjusted to 7.4 ± 0.05 and filtered through 0.22 μm) to dissociate ECP from negatively charged molecules in saliva sample and to avoid losses to the tube walls). Samples were incubated again, for an hour with agitation at room temperature and then centrifuged at 10 000 g for 10 minutes. 100 μL of the supernatant was transferred into 5 mL snap cap tubes for the determination of ECP by UniCAP®.

2.4 | Statistical evaluations

Distributions of the different exposure parameters and salivary biomarkers were first determined using Kolmogorov-Smirnov test. The differences in exposures and biomarker between groups were evaluated using Student's t or Mann-Whitney U tests. The concentration levels of salivary ECP and dust-mite allergens were grouped into tertiles when assessing their associations with doctor diagnosed asthma and current wheeze symptoms.

Multivariate linear regression models were used to analyze the relationship between salivary ECP levels and exposure to mite allergen levels. Since their distributions were found to be not normal, their log-normal concentrations were used in the linear regression analyses.

Chi-square tests were used to compare categorical variables between cases and controls. In comparing the prevalence of health endpoints among different groups, computation of the odds ratios (OR; relative odds) and 95% confidence intervals (95% CI) were performed using logistic regression. Linear-by-linear association was computed to evaluate significant dose-response patterns in crude odds ratios. Multivariate logistic regressions were performed adjusting for potential variables such as gender, age, ethnicity, monthly family income (indicator of socioeconomic status), and family history of asthma.

Statistical analyses were performed using SPSS version 23.0, and significance was evaluated at the conventional 5% level.

3 | RESULTS

Table 1 shows selected demographic, family, and time activity characteristics of the 31 case and 25 control children identified in this study. There were no significant differences between cases and controls for the variables studied.

Mean (median) ECP levels in cases were 66.4 (40.4) mg/L which were significantly higher than those of controls measured at 38.7 (11.3) mg/L ($P = 0.005$; Figure 1). The crude and adjusted odds of a child being labeled as a case increased linearly with higher salivary ECP levels in a dose-response manner (Table 2). Comparing with the first tertile, the odds for asthma were significantly elevated for children with second and third tertile concentrations of ECP.

Blo t 5 allergen levels in the home as well as day-care floors and mattress were almost consistently higher for the cases compared to the controls (Table 3). However, only the differences in day-care floor allergen levels between the cases and controls approached significance ($P = 0.098$). The p value for the differences in time-weighted concentration levels between cases and controls was significant ($P = 0.037$). The greatest time was spent on home mattresses (8.9 hours) and the least was sleeping on day-care mattresses (1.8 h). Hence, Blo t 5 exposure from home mattress residences had the greatest contribution to overall personal exposure while day-care mattress exposure contributed the least.

TABLE 1 Demographic and family characteristics of participating children

	Participating children ^a		
	Total (n = 56)	Controls (n = 25)	Cases (n = 31)
Gender			
Male	30 (53.6%)	13 (52.0%)	17 (54.8%)
Female	26 (46.4%)	12 (48.0%)	14 (45.2%)
Age			
≤3 y old	11 (20.0%)	5 (20.0%)	6 (20.0%)
>3-7 y old	44 (80.0%)	20 (80.0%)	24 (80.0%)
Ethnicity			
Chinese	43 (76.8%)	22 (88.0%)	21 (67.7%)
Malay	9 (16.1%)	2 (8.0%)	7 (22.6%)
Indian	4 (7.1%)	1 (4.0%)	3 (9.7%)
Monthly Family Income ^b			
<\$2 k/mo	5 (9.1%)	0 (0.0%)	5 (16.7%)
\$2 k-3.9 k/mo	19 (34.5%)	11 (44.0%)	8 (26.7%)
\$4-5.9 k/mo	12 (21.8%)	7 (28.0%)	5 (16.7%)
≥\$6 k/mo	19 (34.5%)	7 (28.0%)	12 (40.0%)
Family history of asthma			
Yes	30 (54.5%)	16 (64.0%)	14 (46.7%)
No	25 (45.5%)	9 (36.0%)	16 (53.7%)
Environmental tobacco smoke exposure ^c			
Yes	6 (10.1%)	3 (12.0%)	3 (10.0%)
No	49 (89.1%)	22 (88.0%)	27 (90.0%)
Mean time spent in microenvironments in hours (standard deviation)			
Home floor	5.3 (6.0)	4.2 (5.4)	6.1 (6.4)
Home mattress	8.9 (0.2)	8.9 (0.2)	8.8 (0.2)
Day-care floor	7.2 (0.1)	7.2 (0.2)	7.2 (0.1)
Day-care mattress	1.8 (0.1)	1.8 (0.2)	1.8 (0.1)

^aNumbers may not add up to total because of missing information.

^bMonthly family income in Singapore dollars is used as socioeconomic status (\$1 = 0.73 USD).

^cChild exposed to smoking among family members. Only valid percentages reported.

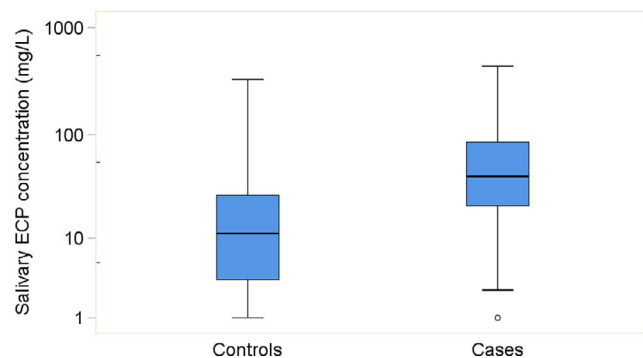
**FIGURE 1** Salivary ECP concentration for cases and controls

Table 4 shows the associations between Blo t 5 allergens in several niches with case status of the children. No statistically significant dose-response pattern was noted for the associations between Blo t 5 concentrations in home and day-care niches with increased odds of

asthma. The 95% confidence intervals for the associations indicated that the odds were all statistically insignificant. Also, no association was observed for asthma with time-weighted Blo t 5 concentrations.

We next compared salivary ECP levels for both case and control subjects with the exposure to allergen concentrations in various microenvironments (Table 5). Although there was a positive relationship between salivary ECP with Blo t 5 levels obtained from the home and day-care environments among the cases, these associations are not statistically significant. However, there was a significant association between salivary ECP levels with time-weighted Blo t 5 concentration. There is no such relationship observed for the controls.

4 | DISCUSSION

This study showed that time-weighted Blo t 5 concentration was associated with salivary ECP levels among asthmatics. This could

TABLE 2 Odds ratios of association between salivary ECP and child classified as doctor diagnosed asthma and current wheeze symptoms by tertiles of salivary ECP

	Salivary ECP			P value for trend ^a
	1st tertile	2nd tertile	3rd tertile	
Concentration (mg/L), mean (inter-quartile range)	2.7 (0.0-12.96)	15.7 (12.97-44.49)	36.2 (44.50-64.0)	
Crude, OR (95% CI)	1.00	3.71 (0.92-15.44)	5.76 (1.32-25.19)	0.017
Adjusted ^b , OR (95%CI)	1.00	5.14 (1.04-25.52)	7.02 (1.38-35.84)	

^aLinear-by-linear association.^bAdjusted for age, gender and ethnicity.

be due to the fact that cumulative Blo t 5 exposure is a predictor for subclinical asthma severity based on biomarkers instead of symptoms. This provides evidence for the hypothesis that the degree of eosinophilic expression through the levels of ECP in the saliva is correlated with the degree of asthma severity.¹⁸ Serum levels of ECP have been shown to correlate with the severity of bronchial asthma¹⁹ and directly correlate with activated eosinophils which are a more sensitive marker of asthma severity than peripheral blood eosinophil counts in acute exacerbations²⁰ sputum and bronchial washings) of asthmatic patients following exposure to diesel particulates.²¹⁻²³ Similar results were obtained in a study by Haldal et al²⁴ dedicated to occupational exposure to bioaerosols with ECP as a marker of chronic airway inflammation and a study by Stoleski et al²⁵ reporting higher salivary ECP levels among adults exposed to environmental tobacco smoke, especially among asthmatics.

Representativeness of one sample is not well documented in literature, but several stationary samples are typically used for a valid exposure assessment.^{1,26} Past studies have reported that allergen concentrations from selected niches correlate poorly with health effects and are probably only surrogates of personal exposures.^{1,27} Our study's findings highlight the importance of considering exposures derived from all of the children's main microenvironments and not just from a single microenvironment. Future exposure assessment protocols should include accurate representations of exposures in homes, day cares, outdoors, and in other indoor settings. Future research should also evaluate whether an allergen avoidance regime in dominant microenvironments of children will have a corresponding reduction effect on their salivary ECP levels. Strategies that could be adopted include air-conditioning to lower relative humidity in the bedrooms and day-care classrooms, removal of mite nests areas such as carpet or unnecessary fabrics and use of effective bed and pillow covers.

We interpret the non-significant increased risk of asthma with wheeze symptoms among children with time-weighted allergen concentrations in comparison to healthy children (Table 4) due to the low sample size (see below) and self-reported questionnaire in our study as possible methodological issues.²⁸ Also, other variables not measured in this study such as medication, diet, and environmental pollution exposure may have influenced the association between exposure and the asthmatic child developing wheeze symptoms.^{29,30}

TABLE 3 Concentrations of Blo t 5 allergens in various microenvironments for cases and controls

	Blo t 5 concentrations in µg/g, median (IQR)		P value
	Controls	Cases	
Home			
Floor	0.20 (0.00-0.62)	0.19 (0.00-1.34)	0.693
Mattress	0.17 (0.00-0.70)	0.41 (0.04-0.72)	0.403
Day care			
Floor	0.11 (0.05-0.22)	0.18 (0.07-0.35)	0.098
Mattress	0.15 (0.11-0.50)	0.23 (0.20-0.83)	0.222
Time-weighted	0.28 (0.14-0.49)	0.39 (0.20-0.82)	0.037 ^a

IQR, inter-quartile range.

^aP < 0.05.

Children are more likely to inhale mite allergens from home mattress dust because of the closer proximity between the nose and mattress during consistent long sleep periods as opposed to those from home floor dusts which may be inhaled after resuspension only during sporadic and vigorous disturbances. The situation is reversed for day-care microenvironments where large number of children involving in various activities could resuspend floor dusts into the breathing zone of children to be inhaled. However, we did not find any significant association between asthma status of the child with allergen exposure from home mattress and day-care floors.

This study provides new evidence that salivary ECP levels are higher in children with asthma than those without, concurring with the results by Schmekel et al¹³ reporting higher saliva ECP levels in asthmatics than in healthy adults. Additionally, our study showed a strong association between salivary ECP levels with and time-weighted environmental Blo t 5 allergen exposure. However, past research has found that salivary ECP in asthmatic children was not associated with asthma severity, concurrence of atopic conditions, medications used, or any skin prick test parameter.¹⁸ Moreover, Bowton et al³¹ found that salivary ECP did not correlate with sputum ECP in adults. Thus, while salivary ECP may not reflect atopy and asthma severity *within* humans, it may reflect activation of eosinophils in oral mucosa by external sources *outside* humans such as asthma-inducing *B tropicalis* allergens. This is not surprising when

TABLE 4 Associations between case status with Blo t 5 allergens

	OR (95% CI)			P value ^a
	1st tertile	2nd tertile	3rd tertile	
Home floor Blo t 5				
Concentration (µg/g)	0.00 (0.00-0.00)	0.20 (0.06-0.62)	1.93 (0.62-6.53)	0.542
Crude ORs (95% CI)	1.0	0.51 (0.14-1.92)	1.69 (0.44-6.45)	
Adjusted ORs (95% CI) ^b	1.0	0.55 (0.14-2.17)	1.28 (0.30-5.35)	
Home mattress Blo t 5				
Concentration (µg/g)	0.00 (0.00-0.09)	0.25 (0.09-0.46)	0.91 (0.49-3.41)	0.594
Crude ORs (95% CI)	1.0	1.39 (0.38-5.07)	1.43 (0.38-5.44)	
Adjusted ORs (95% CI) ^b	1.0	1.28 (0.29-5.62)	1.32 (0.33-5.27)	
Day-care floor Blo t 5				
Concentration (µg/g)	0.07 (0.00-0.13)	0.19 (0.14-0.30)	0.61 (0.33-1.00)	0.455
Crude ORs (95% CI)	1.0	1.72 (0.47-6.32)	1.67 (0.41-6.92)	
Adjusted ORs (95% CI) ^b	1.0	2.03 (0.52-7.87)	2.06 (0.46-9.19)	
Day-care mattress Blo t 5				
Concentration (µg/g)	0.03 (0.00-0.08)	0.13 (0.08-0.19)	0.37 (0.20-0.78)	0.391
Crude ORs (95% CI)	1.0	0.48 (0.12-1.90)	1.92 (0.48-7.77)	
Adjusted ORs (95% CI) ^b	1.0	0.30 (0.06-1.43)	1.71 (0.40-7.37)	
Time-weighted Blo t 5				
Concentration (µg/g)	0.10 (0.00-0.20)	0.34 (0.20-0.52)	1.24 (0.52-3.22)	0.184
Crude ORs (95% CI)	1.0	3.43 (0.83-14.21)	2.75 (0.67-11.24)	
Adjusted ORs (95% CI) ^b	1.0	2.90 (0.66-12.74)	1.91 (0.42-8.77)	

^aLinear-by-linear association.^bAdjusted for age, gender and ethnicity.

one realizes that the oral cavity is connected to the nasopharyngeal cavity and both are constantly exposed to the external environment through breathing.

Unfortunately, sensitization among the subjects was not explicitly evaluated in this particular study. However, we can provide information from our past studies in Singapore. The majority of asthmatic individuals at that age-group are predominately sensitized to both *Dermatophagoides* spp. and *Blomia tropicalis* house dust-mites, specifically more than 90% of asthmatic individuals are house dust-mite

sensitized as assessed by skin prick tests.³ Also, atopic individuals are mostly driven by dominant mono-specific sensitization against house dust-mite in our tropical environment, where the total IgE levels are highly correlated to the house dust-mite specific IgE levels of individuals in Singapore.^{32,33} Koh et al¹⁸ have reported that salivary ECP was not associated with skin prick test (for *Blomia tropicalis*) positivity nor skin prick test wheal size.

This study has several limitations. The small sample size may have led potential selection biases in the study group. For example,

Ln (Blo t 5)	Case/control	β Estimate	SE	t value	P value
Home floor Blo t 5	Control	0.25	0.42	0.58	0.57
	Case	0.16	0.25	0.66	0.52
Home mattress Blo t 5	Control	-0.08	0.40	-0.20	0.85
	Case	0.32	0.27	1.19	0.25
Day-care floor Blo t 5	Control	-0.36	0.37	-0.95	0.35
	Case	0.19	0.35	0.60	0.55
Day-care mattress Blo t 5	Control	-0.02	0.32	-0.05	0.96
	Case	0.28	0.32	0.85	0.40
Time-weighted Blo t 5	Control	0.04	0.28	0.14	0.89
	Case	0.62	0.27	2.34	0.03 ^a

^aP < 0.05; adjusted for gender, age, ethnicity and family history of asthma.**TABLE 5** Multiple linear regression analyses between Ln ECP levels and Ln Blo t 5 levels stratified into cases and controls

participants could be more likely to be from those who are more environmental conscious and/or have more health problems in the case families. Another limitation associated with the low sample numbers is the wide confidence intervals obtained for the associations and non-statistical significant associations. Given the sample size of this study, the calculated observed powers associating case status with salivary ECP and time-weighted Blo t 5 concentrations levels are 0.78 and 0.57, respectively.

5 | CONCLUSIONS

Studies linking dust-mite allergens with asthma and allergic morbidities have typically relied on sampling from representative locations for exposure assessment. We determine the effects of differing microenvironments allergen exposures on asthma and asthma severity among 56 preschool children in Singapore. We found a significant relationship with time-weighted Blo t 5 concentrations with salivary ECP levels among the asthmatics, demonstrating the effects of cumulative exposure on asthma severity. Avoidance measures to reduce Blo t 5 allergen exposure should include all microenvironments that children are exposed throughout the day.

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CONFLICT OF INTEREST

The authors declare there is no conflict of interest arising from this work.

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