

ASTHMA PATHOPHYSIOLOGY

Saliva and Serum Eosinophil Cationic Protein in Asthmatic Children and Adolescents with and without Allergic Sensitization

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Saliva eosinophil cationic protein (ECP) is higher in asthmatic adults than in healthy ones. However, its relationship with asthma severity, other atopic conditions and allergic sensitization have not been reported. Saliva collection is painless, readily acceptable to children and parents, and can be a potentially useful body fluid to measure asthma biomarkers. We recruited 102 physician-diagnosed young asthmatic subjects from outpatient clinics with change in FEV₁ ≥ 12% with inhaled salbutamol and collected data on asthma severity, concurrent allergic rhinitis (AR) and atopic dermatitis (AD), and medication used (bronchodilator and corticosteroid). We measured whole saliva and serum ECP and performed skin prick tests (SPT) for three dust mites (*Dermatophagoides pteronyssinus*, *Dermatophagoides farinae* and *Blomia tropicalis*) and two cockroaches (*Periplaneta americana* and *Blattella germanica*). Median salivary ECP was 84.6 µg/l (inter-quartile range [IQR]: 40.9–147.9) and median serum ECP was 14.7 µg/l (IQR: 6.4–32.1). Serum ECP was only higher in children whose sleep was disturbed by asthma in the past year than those who were not (geometric mean [GM]: 18.9 vs. 11.1 µg/l, relative mean difference [RMD]: 1.71 [95%CI: 1.09–2.69]). Serum ECP was increased among asthmatic children on inhaled corticosteroid than those not using them (GM: 15.6 vs. 8.1 µg/l, RMD: 1.93 [95%CI: 1.11–3.39]). Salivary ECP was not associated with asthma severity, concurrence of atopic conditions, medications used, or any SPT parameter. Saliva ECP did not correlate with serum ECP ($r_s = 0.032$, $p = 0.790$). Salivary and serum ECP did not correlate with wheal size for any skin prick test.

Keywords asthma; atopy; eosinophil cationic protein; saliva; sensitization

INTRODUCTION

The relationship between asthma severity and serum eosinophil cationic protein (ECP) has been well established (1), but the relationship with saliva ECP is relatively understudied. Schmekel et al. found that saliva ECP levels were higher in asthmatics than in healthy adults and decreased with increased doses of inhaled corticosteroids (2). Serum ECP has been shown to correlate with severity of asthma as classified by STEP classification of the National, Heart, Lung, and Blood Institute (NHLBI) (3), severity of atopy based on wheal size and number of positive prick tests (4). However, the relationship between saliva ECP and asthma severity, skin prick test parameters and other atopic conditions such as allergic rhinitis (AR) and atopic dermatitis (AD) have not been reported. Saliva has many advantages as a laboratory test medium because its collection is non-invasive and painless and avoids the risk of needle-stick injuries (5). Hence, it is readily acceptable to children and potentially useful for measuring asthma biomarkers in the young. We compared the relationship between whole saliva and serum ECP, asthma severity, dust mite and cockroach sensitization, and concurrent AR and AD in young asthmatic subjects.

MATERIALS AND METHODS

Subjects

We consecutively sampled physician-diagnosed asthmatics (i.e., patients with recurrent wheezing in the past year who demonstrated a FEV₁ percentage change of >12% after administration of inhaled bronchodilator) 5 to 21 years of age using standardized techniques from a paediatric outpatient clinic (6). Exclusion criteria were acute asthma exacerbation on the day of recruitment and any other chronic lung or heart condition. Informed consent was obtained from parent, and ethics approval was given by the Institutional Review Board.

Questionnaire

We conducted a researcher-administered survey that was modified from the Phase 1 of the International Study of Asthma and Allergies in Childhood (ISAAC) study, designed to evaluate the prevalence and severity of asthma and allergic diseases around the world (7). Demographic data collected included gender, age, ethnicity, and family income. Assessment of asthma severity was based on a series of questions: number of wheezing attacks and hospital admissions in the past year, frequency of coughing or wheezing during the day and night, the effect of asthma on daily activities, attendance at school, and atopy medications used over the past year. We also collected information on the concurrent presence of AR and AD and a family history of asthma, AR, and AD among parents and siblings. AR was defined as frequent paroxysms of sneezing, nasal pruritus, congestion, or profuse watery

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nasal discharge when not having flu in the past one year. AD was defined as chronic or relapsing pruritic rash for more than 6 months in the classical distribution.

Skin Prick Tests (SPT)

We performed SPT on all recruited subjects for dust mites *Dermatophagoides pteronyssinus* (Der p), *Dermatophagoides farinae* (Der f) and *Blomia tropicalis* (Blo t) and cockroaches *Periplaneta americana* (Per a) and *Blatella germanica* (Bla g) according to standard protocol (8). These allergens were chosen because previous studies have found that local children were most commonly sensitized to these dust mite and cockroach species (9). All allergen extracts were obtained commercially from Greer Laboratories, Lenior, N.C., USA except for Blo t, which was prepared in accordance to standard guidelines as previously described (10). The skin of the volar aspect of the forearm was pricked with a disposable lancet with the presence of an allergen droplet. Tests were read at 20 minutes and the diameter of the wheal was recorded in millimeters as a mean (8). The skin prick test was regarded as positive if the mean wheal diameter was 3 mm or greater than the negative control. Glycerol-buffer (25 µg/mL) and 1 mg/mL of histamine was used as negative and positive control subjects, respectively.

Serum ECP

Blood-taking was performed between 11 AM to 4 PM, which is when circadian rhythm of serum ECP is fairly stable (11). Three mL of venous blood was drawn via venipuncture, collected into Serum Separating Tubes (BD Diagnostics, Beller Industrial Estate, Plymouth, PL6 78P, UK), and allowed to clot overnight at 4°C. They were subsequently centrifuged at 3000g at room temperature for 5 minutes. 100-µL aliquots of sera were stored at -70°C until analysis at the end of recruitment. They were transferred into 5-mL snap cap tubes for determination of ECP using UniCAP (Pharmacia Diagnostics, Uppsala, Sweden) according to the manufacturer's instructions.

Saliva ECP

Pure saliva obtained from direct drainage of ducts of major salivary glands is difficult and potentially painful so we opted to collect whole saliva instead, which is more comfortable for children. We followed the laboratory method of Schmekel et al. to analyze saliva ECP (2), except that we added precautionary measures to avoid variable attachment of ECP to test-tube walls and mucus because ECP is a sticky molecule and prone to binding to negatively charged molecules (12). 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 [BD (Becton, Dickinson and Company), USA] which do not bind ECP) 5 minutes later. Saliva was stored at -70°C until analysis. Forty-µL aliquots were diluted to 4:1 with 10% sputolysin (a concentrate of dithiothreitol in phosphate buffer) to isolate epithelial cells. Specimens were vortexed for 30 seconds and then incubated for 15 minutes with agitation at room temperature. Following this, specimens were diluted 2:1 with 0.4% CTAB (N-Cetyl-NNN-trimethylammoniumbromide with pH adjusted to 7.4 ± 0.05 and filtered through 0.22 µm) to dissociate ECP from negatively charged molecules in saliva

TABLE 1.—Characteristics of study population (n = 102).

	Total
Socio-demographic characteristics	(n = 102)
Mean Age (SD) (yr)	11.5 (3.2)
Male gender, n (%)	60 (58.8)
Ethnic group, n (%)	
Chinese	46 (45.1)
Malay	34 (33.3)
Others	22 (21.6)
Monthly household income (tertiles) in S\$, n (%)	
<2,000	36 (35.4)
2,000–3,999	33 (32.3)
>4,000	33 (32.3)
Positive family history in ≥1 first-degree relative, n (%)	
Asthma	67 (65.7)
Allergic rhinitis	74 (72.5)
Atopic dermatitis	41 (40.2)
Asthma severity measures, n (%)	
No. of wheezing attacks in past 1 yr	
None	27 (26.5)
1–3	53 (52.0)
≥ 4	22 (21.5)
No. of admission for asthma in past 1 yr	
None	72 (70.6)
1 or more	30 (29.4)
Frequency of daytime cough or wheezing in past 1 yr	
Never	19 (18.6)
1–2 per wk	74 (72.5)
≥ 3 per wk	9 (8.9)
Frequency of nocturnal sleep disturbance in past 1 yr	
Never	64 (62.7)
1–2 per month	30 (29.5)
≥ 3 per month	8 (7.8)
Effect of asthma symptoms on daily activities	
No	57 (55.9)
Yes	45 (44.1)
Average no. of missed-school days per month in past 1 yr	
Never	68 (66.7)
≥ 1 day	34 (33.3)
Concurrence of atopic conditions, n (%)	
Asthma only	15 (14.7)
Asthma & allergic rhinitis only	48 (47.1)
Asthma & atopic dermatitis only	4 (3.9)
Asthma, allergic rhinitis and atopic dermatitis	35 (34.3)
Medications used regularly in past year, n (%)	
Inhaled bronchodilator	26 (25.5)
Inhaled corticosteroid	72 (70.6)
Nasal corticosteroid	24 (23.5)
Topical corticosteroid	1 (1.0)
Oral corticosteroid	0 (0.0)
Skin prick test positivity, n (%)	
Dust mite allergen	
<i>Dermatophagoides pteronyssinus</i> (Der p)	95 (93.1)
<i>Dermatophagoides farinae</i> (Der f)	94 (92.2)
<i>Blomia tropicalis</i> (Blo t)	89 (87.3)
Cockroach allergen	
<i>Periplaneta americana</i> (Per a)	27 (26.5)
<i>Blatella germanica</i> (Bla g)	44 (43.1)
Eosinophil cationic protein (µg/L), median (inter-quartile range)	
Saliva (n = 91)	84.6 (40.9–147.9)
Serum (n = 71)	14.7 (6.4–32.1)

and to avoid losses to tube walls. Specimens were incubated again for an hour with agitation at room temperature and then centrifuged at 10,000g for 10 minutes. 100 µL of the supernatant was transferred into 5-mL snap cap tubes for the determination of ECP using UniCAP as above.

Statistical Analysis

As the distribution of saliva and serum ECP was skewed, median and inter-quartile values are reported, and

TABLE 2.—Median saliva and serum ECP levels with asthma severity, atopic conditions present, medication used, and skin prick tests.

	Saliva ECP ($\mu\text{g/L}$) (n = 91)			Serum ECP ($\mu\text{g/L}$) (n = 71)		
	n (%)	Geometric mean (95% CI)	RMD (95% CI)	n (%)	Geometric mean (95% CI)	RMD (95% CI)
Gender						
Male	50 (54.9)	90.0 (68.7–120.3)	1.00	39 (54.9)	14.3 (10.4–19.7)	1.00
Female	41 (45.1)	73.7 (55.7–97.5)	0.82 (0.55–1.21)	32 (45.1)	13.2 (9.4–18.5)	1.09 (0.58–1.46)
Ethnicity						
Chinese	43 (47.3)	73.7 (55.1–97.5)	1.00	30 (42.2)	12.3 (8.24–18.4)	1.00
Malay	30 (33.0)	83.1 (59.1–117.9)	1.13 (0.73–1.76)	26 (36.6)	15.6 (10.8–22.6)	1.27 (0.97–2.18)
Others	18 (19.8)	105.6 (66.0–169.0)	1.20 (0.92–1.56)	15 (21.1)	13.7 (8.7–21.8)	1.06 (0.77–1.45)
Monthly household income (S\$)						
<2,000	31 (34.1)	75.9 (56.3–101.5)	1.00	25 (35.2)	15.3 (10.0–23.3)	1.00
2,000–3,999	32 (35.2)	97.5 (67.4–114.2)	1.29 (0.80–2.06)	24 (33.8)	14.3 (10.0–20.7)	0.94 (0.54–1.62)
>4,000	28 (30.8)	74.4 (51.4–106.7)	0.99 (0.79–1.24)	22 (31.0)	11.7 (7.5–18.5)	0.88 (0.65–1.45)
Measures of asthma severity						
No. of wheezing attacks in past year						
0–3	71 (78.0)	77.5 (61.6–97.5)	1.00	55 (77.5)	13.3 (9.3–13.3)	1.00
4 or more	20 (22.0)	102.5 (68.7–151.4)	1.11 (0.76–1.65)	16 (22.5)	15.6 (9.2–26.6)	1.22 (0.76–1.95)
No. of admission for asthma in past year						
None	64 (70.3)	85.6 (68.0–108.9)	1.00	51 (71.8)	13.2 (10.0–17.5)	1.00
1 or more	27 (29.7)	75.2 (51.9–107.8)	0.87 (0.57–1.34)	20 (28.2)	15.5 (10.0–23.8)	1.17 (0.70–1.95)
Frequency of daytime cough or wheezing in past year						
Never	15 (16.7)	85.6 (49.4–148.4)	1.00	10 (14.1)	11.2 (6.5–19.3)	1.00
≥ 1 per wk	75 (83.3)	81.5 (66.0–100.5)	1.11 (0.85–1.45)	60 (85.7)	14.3 (11.0–18.4)	1.16 (0.86–1.58)
Frequency of nocturnal sleep disturbance in past year						
Never	56 (62.2)	81.5 (63.4–103.5)	1.00	42 (59.2)	11.0 (8.3–14.7)	1.00
≥ 1 per month	34 (37.8)	83.9 (60.3–116.7)	1.03 (0.69–1.54)	28 (39.4)	18.9 (13.2–27.1)	1.71 (1.09–2.69)*
Effect of asthma symptoms on daily activities						
No	50 (54.9)	79.8 (61.6–104.6)	1.00	35 (49.3)	13.1 (9.1–18.7)	1.00
Yes	41 (45.1)	84.8 (62.8–114.4)	1.06 (0.72–1.57)	36 (50.7)	14.4 (10.7–19.5)	1.11 (0.70–1.75)
No. of missed schooldays per month in past year						
Never	61 (67.0)	89.1 (69.4–114.4)	1.00	48 (67.6)	13.7 (10.3–18.4)	1.00
≥ 1 day	30 (33.0)	70.1 (51.4–95.6)	0.79 (0.52–1.20)	23 (32.4)	13.7 (9.3–20.3)	1.00 (0.61–1.63)
Concurrence of atopic conditions						
Asthma only	15 (16.5)	72.2 (38.9–134.3)	1.00	12 (16.9)	16.6 (9.0–30.9)	1.00
Asthma & allergic rhinitis only	42 (46.2)	111.1 (85.6–142.6)	1.53 (0.89–2.64)	32 (45.1)	16.0 (11.6–22.0)	0.96 (0.52–1.79)
Asthma & atopic dermatitis only	4 (4.4)	34.1 (4.1–287.1)	0.69 (0.89–1.38)	2 (2.8)	6.3 (1.0–49.4)	0.61 (0.28–1.34)
Asthma, allergic rhinitis and atopic dermatitis	30 (33.0)	65.4 (47.9–88.2)	0.97 (0.79–1.17)	25 (35.2)	11.0 (7.2–17.3)	0.87 (0.68–1.11)
Medications used regularly in past year						
Inhaled bronchodilator						
Irregularly (less than daily)	67 (73.6)	86.5 (68.7–110.0)	1.00	50 (70.4)	13.3 (10.2–17.3)	1.00
Regularly (daily)	24 (26.4)	71.5 (49.9–101.5)	0.82 (0.53–1.28)	21 (29.6)	15.0 (9.3–24.3)	1.14 (0.8–1.88)
Inhaled corticosteroid						
No	19 (20.9)	62.8 (42.9–92.8)	1.00	14 (29.6)	8.1 (5.1–12.8)	1.00
Yes	72 (79.1)	88.2 (70.8–111.1)	1.40 (0.87–2.25)	57 (70.4)	15.6 (12.2–20.3)	1.93 (1.11–3.39)*
Nasal corticosteroid						
No	33 (45.8)	77.5 (58.0–103.55)	1.00	56 (78.9)	11.8 (8.4–16.6)	1.00
Yes	39 (54.2)	99.5 (75.2–133.0)	1.28 (0.86–1.92)	15 (21.1)	16.0 (10.7–24.0)	1.36 (0.81–2.27)
Skin prick test positivity (n = 107)						
Dust mite allergen						
<i>Dermatophagoides pteronyssinus</i> (Der p)						
No	7 (7.7)	73.0 (31.5–169.0)	1.00	5 (7.0)	6.9 (3.3–14.6)	1.00
Yes	84 (92.3)	68.0 (68.0–101.5)	0.88 (0.42–1.82)	66 (93.0)	14.6 (11.5–18.4)	2.10 (0.87–5.05)
<i>Dermatophagoides farinae</i> (Der f)						
No	7 (7.7)	53.0 (18.5–152.9)	1.00	4 (5.6)	6.5 (2.2–19.3)	1.00
Yes	84 (92.3)	85.6 (70.1–103.5)	0.63 (0.30–1.28)	67 (94.4)	14.4 (11.4–18.2)	2.20 (0.84–5.87)
<i>Blomia tropicalis</i> (Blo t)						
No	12 (13.2)	66.0 (37.0–119.1)	1.00	7 (9.9)	9.4 (3.8–22.9)	1.00
Yes	79 (86.8)	84.8 (68.7–104.6)	1.28 (0.73–2.29)	64 (90.1)	14.4 (11.4–22.9)	1.54 (0.71–3.29)
Cockroach allergen						
<i>Periplaneta americana</i> (Per a)						
No	64 (70.3)	84.8 (66.0–107.8)	1.00	49 (69.0)	13.5 (10.3–17.8)	1.00
Yes	27 (29.7)	77.5 (56.8–105.6)	0.92 (0.60–1.40)	22 (31.0)	14.3 (9.3–22.2)	1.06 (0.64–1.75)
<i>Blattella germanica</i> (Bla g)						
No	51 (56.0)	80.6 (60.3–107.0)	1.00	37 (52.1)	13.3 (9.8–18.0)	1.00
Yes	40 (44.0)	84.8 (64.7–111.1)	1.05 (0.71–1.57)	34 (47.9)	14.3 (10.0–20.5)	1.08 (0.68–1.72)

* $p < 0.05$.

RMD = relative mean difference; ECP = eosinophil cationic protein.

logarithmic transformation was performed to render them parametric. One-way analysis of variance (ANOVA) was used to test for associations between categorical independent variables, and saliva or serum ECP levels as dependent variables, and geometric means and relative mean difference

(RMD), which is the ratio of two geometric means, are reported. Spearman rank correlation (r_s) was used to determine the relationship between saliva and serum ECP and with skin prick wheal size. Multiple linear regression was used to determine the independent predictors of saliva and serum log ECP

TABLE 3.—Correlation between age, skin prick wheal size and saliva and serum ECP levels.

	Saliva ECP (n = 91)		Serum ECP (n = 71)	
	Spearman correlation (r_s)	p value	Spearman correlation (r_s)	p value
Age (yrs)	0.171	0.087	0.049	0.669
Skin prick wheal size (n = 107)				
Dust mite allergen				
<i>Dermatophagoides pteronyssinus</i> (Der p)	−0.15	0.13	0.08	0.49
<i>Dermatophagoides farinae</i> (Der f)	0.06	0.54	0.26	0.02
<i>Blomia tropicalis</i> (Blo t)	0.12	0.22	0.17	0.15
Cockroach allergen				
<i>Periplaneta americana</i> (Per a)	0.04	0.70	0.17	0.14
<i>Blattella germanica</i> (Bla g)	0.09	0.36	0.19	0.10

values. We used SPSS version 14.0 (SPSS Inc, Chicago, IL, USA) for statistical analysis, and significance was evaluated at the conventional 5% level.

RESULTS

A total of 102 of 107 (95.3%) eligible subjects gave consent for participation. All subjects underwent SPT, 71 provided serum, and 91 provided saliva samples. Table 1 reports the socio-demographic characteristics, history of atopy in first degree relatives, self-reported measures of asthma severity, concurrence of atopic conditions, medications used regularly in past year, positive skin prick test results to dust mite and cockroach allergens tested, and median saliva and serum ECP levels in the study population.

Table 2 presents the geometric means of saliva and serum ECP levels for socio-demographic variables (except age), asthma severity, concurrent atopic conditions, medications used, and SPT positivity. Saliva and serum ECP were not associated with age (Table 2), gender, ethnicity, or monthly household income. Subjects with nocturnal sleep disturbance from asthma symptoms in the past year had higher serum ECP levels than those who did not (geometric mean: 18.9 vs. 11.0 $\mu\text{g/L}$, RMD: 1.71 [95%CI: 1.09–2.69]). Subjects on inhaled corticosteroids also had higher serum ECP than those not on them (geometric mean: 15.6 vs. 8.1 $\mu\text{g/L}$, RMD: 1.93 [95%CI: 1.11–3.39]). On multivariate analysis, only use of inhaled corticosteroids was independently associated with serum ECP. Salivary ECP was not associated with asthma severity or atopy medications used. Both serum and salivary ECP were not associated with concurrence of atopic conditions, skin prick test positivity or wheal size (Table 3). Saliva ECP did not correlate with serum ECP ($r_s = 0.032$, $p = 0.790$).

DISCUSSION

Similar to previous studies on serum ECP and asthma severity, we also found that it was associated with nocturnal sleep disturbance from asthma symptoms and use of inhaled corticosteroids. However, unlike the study of Schmekel et al. (2), we found no association between saliva ECP and asthma severity, atopy medications used, concurrence of atopic conditions, skin prick test positivity or wheal size, or serum ECP. A possible reason for the different findings could be that saliva ECP levels are less discriminatory as a biomarker in the younger age group than in adults. While our study does not support the use of saliva ECP as an assessment tool for asthma and atopy, saliva still remains a potentially useful

biological fluid because of its ease of collection. Saliva has an abundance of proteins and electrophoresis-based peptide separation, and tandem mass spectrometry has identified at least 437 proteins of various biological functions (13). While certain biomarkers have been found to be raised in saliva of asthmatics such as thromboxane B2, leukotriene B4, and prostaglandin F2a (14), IgE (15), and peroxidase activity (16), their usefulness in assessing asthma severity or control has yet to be tested. With the integration of proteomics with clinical asthma research, there is great potential for identification of saliva biomarkers of asthma, which may objectively assess asthma control or predict relapse in controlled asthmatics.

This study has several limitations. The small sample size may have led to the lack of any association between saliva ECP and parameters of asthma and atopy. For example, based on sample size calculations, the difference in saliva ECP between those on inhaled corticosteroids and those not on would have been statistically significant if there were at least 133 subjects who had their saliva ECP measured. Another limitation is the lack of a control group, which may provide more information on the relationship of saliva ECP between asthmatics and non-asthmatics. This was a cross-sectional study so it lacked information on the value of serial changes in saliva ECP, which may be more useful in assessing asthma control. Lastly, it would have been useful to include adults in the study to compare the saliva ECP levels and its relationship with asthma and atopy parameters between older and younger asthmatics.

In conclusion, among young asthmatics, serum ECP is associated with nocturnal sleep disturbance from asthma symptoms and use of inhaled corticosteroids. However, saliva ECP is not associated with asthma severity, atopy medications used, concurrence of atopic conditions, SPT positivity or wheal size, or serum ECP and does not appear to be useful as an assessment tool for asthma and atopy.

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DECLARATION OF INTEREST

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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