Original article

A study of club (Clara) cell protein (CC16) expression in a group of atopic asthmatic children

Background*: The dysregulation of CC16 protein secreted by club cells (Clara cells) was reported in acute respiratory distress syndrome and Chronic obstructive pulmonary disease. We sought to investigate serum and urinary CC16 in asthmatic children in relation to asthma exacerbation and quiescence and correlate it to pulmonary function test results.* ***Methods:*** *This prospective controlled study was conducted in the Pediatric Allergy, Immunology and Rheumatology Unit, Children's Hospital, Ain Shams University on 40 atopic asthmatic patients, 6-12 years old, and 40 matched healthy controls, during the period from March 2020 to August 2021. Patients were enrolled consecutively during an asthma flare and were followed up to be re-evaluated at asthma quiescence. Patients were subjected to clinical assessment, skin prick testing to common aeroallergens, pulmonary function testing, and measurement of serum and urinary CC16 by ELISA during asthma exacerbation and quiescence.* ***Results:*** *Serum and urinary CC16 levels in patients during asthma exacerbation (median 243.5 and 137.5 ng/ml, respectively) and quiescence (median 112.5 and 55 ng/ml) were significantly higher than the levels in matched controls (median 15 and 13 ng/ml). Moreover, serum and urinary CC16 levels were higher during exacerbation than during quiescence (z=-5.214 and 4,941 respectively, p values < 0.001). Urinary CC16 showed significant inverse correlation with best FVC% of predicted (r=-0.408, p= 0.009), but no significant correlation was found with age, BMI, age of onset of asthma, disease duration.* ***Conclusion:*** *Serum and urinary CC16 levels seem to be elevated in asthmatic children especially during asthma exacerbation. Measurement of CC16 in urine might represent a non-invasive option that can replace blood sampling. Further wider scale studies involving timely measurements of CC16 are needed to efficiently assess the role of CC16 as a biomarker of asthma exacerbation.*

Keywords: Clara cell protein, atopic, asthma, exacerbation, biomarker

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INTRODUCTION

Club cells formally called Clara cells, are non-ciliated bronchiolar secretory cells in the respiratory epithelium.[1] Club cell protein (CC16) is secreted by the non-ciliated Clara cells and by non-ciliated columnar cells of the large and small airways.[2] Although the exact role of CC16 is still not well known, it has been shown to have anti-inflammatory and anti-oxidative role in various cells.[3,4] CC16 has immunomodulatory effects and suppresses phospholipase A2, thus decreasing the production of eicosanoids.[5] Clara cells express the IL-4 cytokine receptor[6] and secret pro- and anti-inflammatory factors that modulate immune responses.[7] They play a key role in the biotransformation of inhaled xenobiotics together with cytochrome P-450 and mixed-function mono-oxygenases. For this reason, club cells are believed to play an important role in protecting the airways from the harmful influence of a toxic external environment.[8] Club cells also protect and regulate pulmonary function through the secretion of surfactants, glycosaminoglycans, enzymes, and other proteins. CC16 attenuates surfactant degradation and oxidative stress.[9,10]

Respiratory disorders that increase the epithelial permeability (e.g., acute bronchiolitis,[11] acute respiratory distress syndrome,[12] exposure to proinflammatory agents,[13] idiopathic pulmonary fibrosis,[14] bronchopulmonary dysplasia[15] have been associated with higher serum levels of CC16.

Studies of CC16 in asthma, particularly in children, are limited with contradictory results. The CC16 gene is located on chromosome 11q 12–13, a region that has been associated with asthma and atopy [16] and polymorphism of the CC16 gene was suggested to increase the risk of developing

asthma.[17] With the scant data on CC16 expression in asthmatic children, we were stimulated to carry out this study, aiming to investigate the role of CC16 in pediatric asthma, its relation to asthma exacerbation and value in follow-up of asthmatic children.

METHODS

This prospective controlled study was conducted in the Pediatric Allergy, Immunology and Rheumatology Unit, and the Pediatric Pulmonology Unit, Children's Hospital, Ain Shams University in the period from March 2020 to August 2021. The study was conducted after approval of the Research Ethics Committee of Ain Shams University Hospitals [approval number FMASU-MD: 228/2020]. An informed consent was obtained from the parents or caregivers of each subject prior to enrollment.

Study population

Patients' group (n=40) included 6-12 years old children with physician diagnosed asthma, defined according to GINA guidelines with verified atopy (positive skin prick test to one or more of the common environmental aeroallergens). We excluded patients with any clinical evidence of chronic illness other than asthma. Patients were enrolled consecutively during asthma flare ups after fulfilment of the inclusion criteria and were followed up till asthma quiescence, with clinical and laboratory evaluation during asthma exacerbation and quiescence. Asthma exacerbation and quiescence were defined according to clinical assessment. Evaluation during asthma quiescence was performed at least 1 month after the last asthma exacerbation. Age and gender matched controls (n=40) were recruited from the Outpatient Clinics including healthy children presenting with minor traumatic or surgical problems after exclusion of any chronic illness or atopy. The latter was judged by the absence of personal or family history of allergy and through skin prick testing**.** Controls were evaluated once at enrolment.

Sample size

We recruited 40 patients and 40 healthy matched children. A sample size of at least 34 data pairs achieves 80.8% power to reject the null hypothesis of zero effect size when the population effect size is 0.50 (a medium sized effect) and the significance level (alpha) is 0.050 using a two-sided paired t-test.[18] This sample size is satisfactory to detect a two-sided 95% confidence interval with a width equal to 0.4 when the estimate of Pearson's product-moment correlation between Clara level and pulmonary function tests is 0.70

Study methods

All enrolled patients were subjected to the following:

History taking with special emphasis on the age of onset of asthma and duration of the disease, family history of allergic diseases, other allergic manifestations (skin allergy, allergic rhinitis, anaphylaxis), previous hospital admission, triggers and potentiators of asthma exacerbation, and therapeutic history especially for inhaled and/or systemic steroids.

Clinical examination at enrollment and after quiescence of symptoms including general examination including the growth parameters in relation to normal centiles for age (CDC Growth Charts, 2000), detailed chest examination for signs of respiratory distress and degree of asthma exacerbation and complete systemic examination for other sites of allergy like allergic shiner, transverse nasal crease, eczema, and urticarial rash.

Investigations

1. Skin prick testing using standardized allergen extracts (Omega Laboratories, Montréal, Canada) of common environmental aeroallergen (mite mix, aspergillus, cockroach, cat dander and pollens) to judge atopy in patients and controls at enrolment

2. Pulmonary function tests: Forced spirometry was performed during asthma exacerbation by a pulmonologist in the Pediatric Pulmonology Unit, Children's Hospital, Ain Shams University using JAEGER apparatus (Care Fusion Germany, 2011). The apparatus used meets the American Thoracic Society standards and uses up-to-date predicted values based upon age, sex, ethnicity, height, and weight.[19] The following variables were obtained from the best of 3 reproducible forced expiratory manoeuvres: FVC (forced vital capacity), FEV1 (forced expiratory volume in one second), FEV1/FVC (%) and forced expiratory flow between 25% and 75% expired volumes (FEF 25-75%). Results were interpreted according to Barreiro and Perillo.[20]

3. Club cell protein (CC16): The Human Clara cell (Club cell) protein (CC16) was measured in serum and urine during flare up and after quiescence of asthma manifestations and once in controls. CC16 concentration was determined using Human Clara Cell Protein ELISA kits from Bioassay technology lab, China according to the manufacturer instructions.

Statistical Analysis

Statistical analysis was done using MedCalc® Statistical Software version 20 (MedCalc Software Ltd, Ostend, Belgium; https://www.medcalc.org; 2021) and IBM© SPSS© Statistics version 26 (IBM© Corp., Armonk, NY). Categorical variables are presented as numbers and percentages and intergroup differences are compared using the Pearson chi-squared test. Numerical data are presented as median and interquartile range (IQR) and between-group differences are compared using the Mann-Whitney U-test (for 2-group comparison). Correlations between numerical variables are tested using the Spearman rank correlation or Pearson correlation.

RESULTS

The study comprised 40 atopic asthmatic and 40 matched healthy controls. The age of enrolled patients ranged between 6 and 12 years with median of 9 years (IQR: 6-11). We observed a male predominance among patients with male to female ratio 3:1. BMI of our patients ranged from 10.8 to 31 with median of 16.64 (IQR: 14.66-20). Asthma characteristics among the enrolled patient is shown in table (1). The most common sensitizing allergens found was mite mix (31 patients; 77.5%) followed by cat dander (n= 30; 75%), cockroach (n= 22, 55%), aspergillus and pollen mix (each n= 21, 52.5%).

CC16 levels were significantly higher among asthmatics in exacerbation in both urine and serum as compared to controls (table 2 and figures 1 and 2). Quiescence levels were also significantly higher in patients compared to healthy controls as shown in table 3 and figures 3 and 4. Furthermore, CC16 levels during exacerbation of asthma were significantly higher as compared to quiescence levels as shown in table 4. However, different grades of severity of asthma exacerbation (mild, moderate, and severe) showed comparable levels of urinary and serum CC16 (using Jonckheere-Terpstra trend test: p= 0.957 and 0.929, respectively).

Urinary CC16 levels during asthma exacerbation showed significant inverse correlation with best FVC% of predicted (r= -0.408, p= 0.009) (figure 5). Exacerbation levels of urinary CC16 also correlated negatively with cockroach wheal diameter (r= -0.321, p= 0.044) and positively with cat wheal diameter (r=0.473, p= 0.002) on skin prick test. No significant correlation was found between urinary and serum CC16 levels and the other pulmonary functions parameters or clinicodemographic variables (including age, weight and height centiles, BMI, and asthma duration). Serum CC16 did not show significant correlation to urinary CC16, neither during asthma exacerbation (r=0.192, p=0.235) nor during quiescence (r=-0.207, p= 0.2). However, using simple linear regression model, serum CC16 during asthma exacerbation could be estimated from urinary CC16 with a precision of ± 1.578 ng/ml (Log SEest = 0.198 ng/ml, SEest = 1.578 ng/ml) (t= 3.188, p= 0.003) (figure 6). Also, serum CC16 during asthma quiescence, could be estimated from urinary CC16 with a precision of ± 1.479 ng/ml (Log SEest = 0.170 ng/ml, SEest = 1.479 ng/ml) (t= -3.000, p= 0.005) (figure 7).

Table 1. Clinical data of asthmatic patients (n=40)

	Range [Median (IQR)] or No (%)		
Known/newly diagnosed asthma	Known		15 (37.5%)
	New		25 (62.5%)
Duration of asthma (years)	1 to 11		
	[4 (3-6)]		
Exacerbation severity	Mild		5 (12.5%)
	Moderate		33 (82.5%)
	Severe		2 (5%)
Treatment received	ICS	Low dose	1 (2.5%)
		Medium dose	9 (22.5%)
		High dose	1 (2.5%)
		No	29 (72.5%)
	LABA (with ICS)		5 (12.5%)
	LTRA		9 (22.5%)

Other allergic manifestation	Skin allergy	1 (2.5%)
	Airway allergy	17 (42%)
	No	22 (55%)
Suspected asthma exacerbation trigger	Aeroallergen	7 (17.5%)
	Infections	4 (10%)
	Food	0
	Exercise	1 (2.5%)
	Unknown	28 (70%)
Exacerbation management	ER/Ward admission	27 (67.5%)
	PICU admission	0
	Home management	13 (32.5%)
Family history	Positive	28 (70%)

ER: emergency room, ICS: inhaled corticosteroids, LABA: long acting beta2 agonist, LTRA: leukotriene receptor antagonist, PICU: pediatric intensive care unit

Table 2. Urinary and serum CC16 during asthma exacerbation versus controls

	Cases (n=40)		Controls (n=40)		Mann-Whitney U test		
Variable	Median	IQR	Median	IQR	U	Z	p-value
Urinary CC16 during exacerbation (ng/ml)	137.5	112.5 to 181.0	13.0	12.5 to 25.0	0.5	7.710	<0.0001
Serum CC16 during exacerbation (ng/ml)	243.5	170.0 to 306.25	15.0	12.5 to 25.0	0.0	7.723	<0.0001

IQR = interquartile range, U = U-statistic, z = Z-statistic.

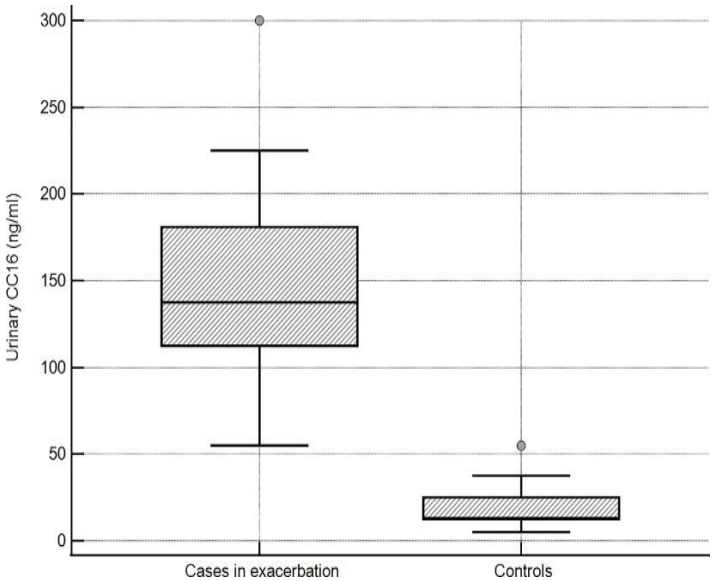

Figure 1. Box plot illustrating urinary CC16 in cases during exacerbations versus controls.
Box represents the interquartile range (25th to 75th percentile). Line inside the box represents the median (50th percentile). Whiskers represent the minimum and maximum values excluding outliers (rounded markers).

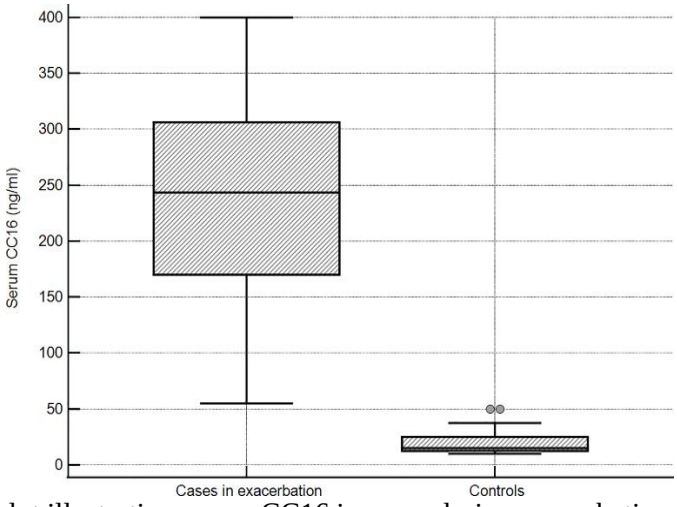

Figure 2. Box plot illustrating serum CC16 in cases during exacerbation versus controls.
Box represents the interquartile range (25th to 75th percentile). Line inside the box represents the median (50th percentile). Whiskers represent the minimum and maximum values excluding outliers (rounded markers)

Table 3. Urinary and serum CC16 levels during asthma quiescence versus controls

	Cases (n=40)		Controls (n=40)		Mann-Whitney U test		
Variable	Median	IQR	Median	IQR	U	Z	p-value
Urine CC16 during quiescence (ng/ml)	55.0	37.5 - 75.0	13.0	12.5 - 25.0	55.0	7.185	<0.0001
Serum CC16 during quiescence (ng/ml)	112.5	75.0 - 135.5	15.0	12.5 - 25.0	11.5	7.614	<0.0001

CC16: Clara cell protein; IQR = interquartile range; U = U-statistic; z = Z-statistic

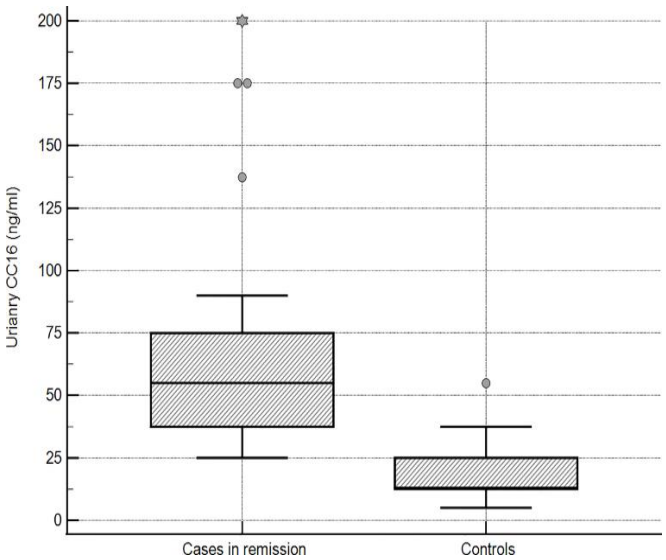

Figure 3. Box plot illustrating urinary CC16 in cases during asthma quiescence versus controls**.**
Box represents the interquartile range (25th to 75th percentile). Line inside the box represents the median (50th percentile). Whiskers represent the minimum and maximum values excluding outliers (rounded markers) and extreme values (asterisks)

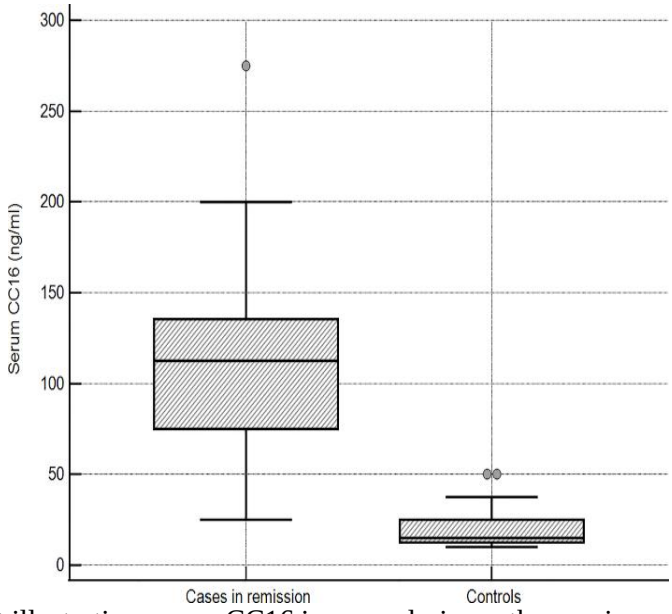

Figure 4. Box plot illustrating serum CC16 in cases during asthma quiescence versus controls.
Box represents the interquartile range (25th to 75th percentile). Line inside the box represents the median (50th percentile). Whiskers represent the minimum and maximum values excluding outliers (rounded markers)

Table 4. Serum and urinary CC16 levels in asthma exacerbation versus quiescence.

	Cases in exacerbation (n=40)		Cases in quiescence (n=40)		Wilcoxon Ranks signed test	
Variable	Median (IQR)	range	Median (IQR)	Range	Z	p-value
Urinary CC16 (ng/ml)	137.5 (112.5 – 181.0)	55 – 300	55.0 (37.5 – 75.0)	25 – 200	7.710	<0.0001
Serum CC16 (ng/ml)	243.5 (170.0 − 306.3)	55 – 400	112.5 (75 − 135.5)	25 – 275	7.723	<0.0001

CC16: clara cell protein, IQR = interquartile range, z = Z-statistic.

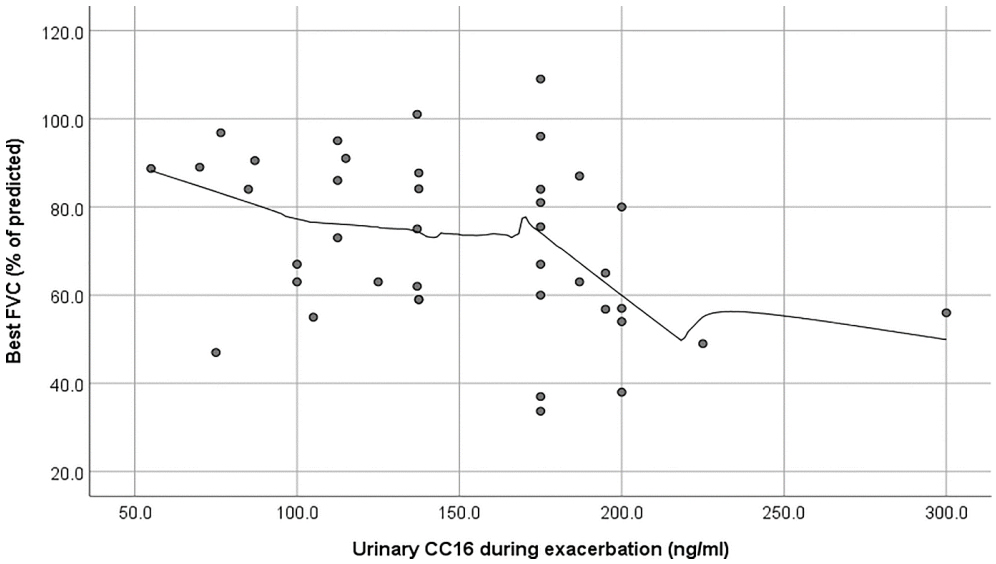

Figure 5. Scatter plot illustrating the correlation between urinary CC16 during exacerbation and best FVC (r= -0.408, p= 0.009).
Fitted line represents the locally estimated scatterplot smoothing (LOESS) function.
CC16: Clara cell protein, FVC: forced vital capacity.

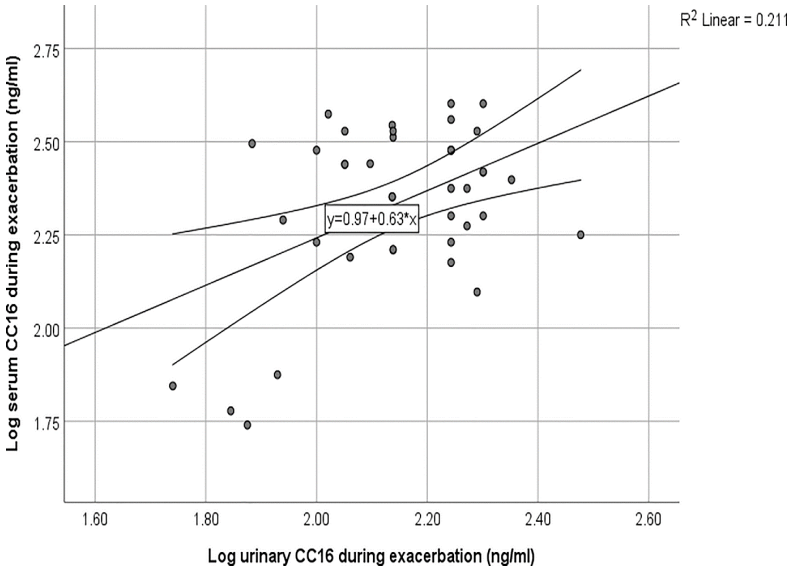

Figure 6. Scatter plot illustrating the correlation between urinary and serum CC16 during exacerbation.
Fitted lines represents the linear regression line with its 95% confidence limits (95% CI)

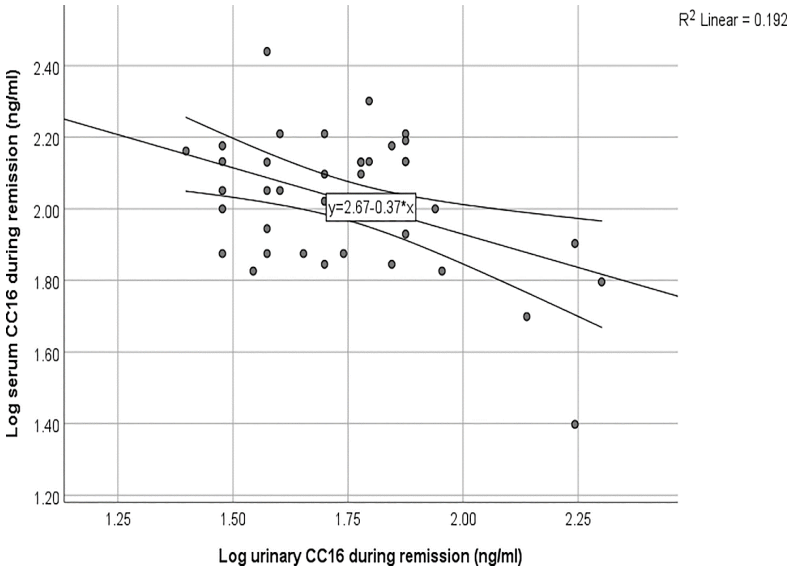

Figure 7. Scatter plot illustrating the correlation between urinary and serum CC16 during remission.
Fitted lines represents the linear regression line with its 95% confidence limits (95% CI)

DISCUSSION

Many researchers studied CC16 level in various body fluids; in serum, bronchoalveolar lavage, sputum, and nasal fluid. Few studies were conducted on urinary CC16 and in relation to atopic asthma. This study was aimed to investigate serum and urinary CC16 expression in a group of asthmatic children during exacerbation and quiescence, in relation to pulmonary functions, in a trial to estimate its possible role in asthma pathogenesis and in clinical management.

Our study showed that urinary CC16 levels were higher during asthma exacerbation as compared to the control group. Levels during asthma quiescence were still higher than the control values. These findings outline a possible role for CC16 as a marker of atopy and/or asthma.

To the best of our knowledge only one study investigated urinary CC16 in asthmatics. It was conducted on 147 asthmatic Chinese children 9-15 years and 390 healthy controls. However, this study reported decreased urine CC16 levels in association to asthma and impeded lung functions.[21] The contradiction between their observation and ours may be attributed to variation in the studied samples in term of age, race, and atopy. Our smaller sample contained children with persistent as well as intermittent symptoms and was not consistent in terms of therapy received or degree of control. Our findings could have been also influenced by the

presence of concomitant respiratory infections or exposure to tobacco smoke and both of them could alter CC16 levels. The latter is very common in our community. Another possible explanation is that Ma et al used morning midstream samples and measured the CC16 in relation to urinary creatinine level rather than measuring absolute values as we did. CC16 has diurnal variation and urine samples in our study were collected at variable times.

Although we did not investigate for viral infections in our asthmatic patients during exacerbation, yet the possibility of the presence of underlying viral triggers is present and might have participated to the elevation of urinary CC16 level in our patients, particularly during asthma exacerbation. Johansson and colleagues found that infants with RSV infection had significantly higher serum CC16 levels compared with healthy controls but not with influenza or parainfluenza virus.[22] More recently, Egron et al. found a urinary increase in CC16 correlating to the severity of acute bronchiolitis in infant under 1 year.[12]

Gioldassi et al[23] found significantly lower levels of CC16 in the serum of 24 asthmatic Greek children aged 0-14 years as compared to 27 age matched healthy ones. A study from Japan on 63 Japanese adult nonsmokers with chronic persistent asthma (37 were atopic and 26 non atopics) revealed that serum CC16 levels in adult asthmatic nonsmokers are significantly decreased compared with healthy nonsmokers. Worth mentioning is that the blood samples were collected from the 63 patients when they did not have an asthmatic attack and from 10 of the 63 patients at the time of the asthmatic attack.[24]

In bronchoalveolar lavage and sputum, results of CC16 were controversial. One study reported lower CC16 levels in bronchoalveolar lavage (BAL) fluid from 24 patients with asthma (mean age, 40±3.1 years) compared with 24 controls (mean age, 29 ±11 years).[25] Another found higher CC16 levels in the sputum of asthmatics (n=84) compared to controls (n=12).[26] In contrast, a study on 32 patients with asthma (24 of them were atopic) and 19 control subjects and reported no difference in sputum CC16 levels of atopic asthmatics and controls.[3]

Urinary CC16 was higher during asthma exacerbation compared to quiescence suggesting potential role of urinary CC16 as a non-invasive biomarker of asthma exacerbation. Subsequent measurement of CC16 may be needed in a sequential pattern to determine the variation in its level in relation to bronchospasm and the possibility of its use to predict asthma exacerbation before clinical manifestations, with possible adjustment of asthma treatment accordingly aiming to reach optimum asthma control. Further studies are needed to validate our assumptions. Shijubo et al found no significant difference of serum CC16 levels at the time of acute attack (n=10) and in stable condition in their study that was conducted on 63 asthmatic adults. They considered their observation limited by the sample size.[24]

There are several factors that might contribute to the variability of results among different studies, including but not limited to: the degree of exposure to pollutants[27] especially tobacco smoking,[28] the relation to exercise[29] and diurnal variation in CC16 itself.[30] The presence of underlying infection[26] even if subclinical could be another confounding factor. Within the allergic disease itself, the different phenotypes, the severity, control of symptoms, the atopic status and medications[31] received might have their impact as well.

In our series, there was no statistically significant difference between urinary CC16 levels in relation to the severity of asthmatic exacerbation. Also, we observed that level of urinary CC16 during asthma exacerbation is inversely correlated with best FVC% of predicted but not with best FEV1% of predicted or FEV1/FVC. These findings might denote that its role in predicting the severity of asthma exacerbation or in reflecting the degree of airway obstruction is limited. However, the small sample size and the difficult technique of pulmonary function tests especially that it was performed during exacerbation with the possibility of air trapping. In this aspect, a relevant study revealed that plasma CC16 levels were positively correlated with FEV1%, but not with FVC% nor FEV1/FVC ratio.[32] However, they noted that decreased plasma CC16 was associated with airflow limitation and damaged lung functions and reported a negative correlation between plasma CC16 levels and bronchial hyperresponsiveness in asthmatics.

A positive association was reported between serum CC16 levels and FEV1% predicted and FEV1/FVC.[33] Experimental acute damage to club cells could cause reduction in club cell numbers, CC16 mRNA, and protein levels in the lung with significant increase in serum concentration due to leakage of the bronchoalveolar/blood barrier.[34] It is, therefore, possible that a transient elevation of serum and consequently urinary CC16 might occur with asthma exacerbation. Sequential measurement of CC16 on close intervals might demonstrate more

clear results. A study on rats reported decreased serum CC16 levels after an initial transient increase compared with control rats exposed to chemicals.[35] Worth to note that we observed that CC16 levels during asthma quiescence were still higher than controls. Whether this is due to an underlying subclinical Clara cell damage and continued inflammation or not, needs further assessment.

We observed that CC16 exacerbation levels, whether in serum or urine, were not affected by age, weight, or BMI. In a previous study, serum CC16 levels were found to increase in obesity and levels showed significant positive correlation with triglycerides, cholesterol, apo A-I, apo A-II, apo B, and BMI, and an inverse correlation with HDL.[36] In another study, no influence of serum lipids, BMI, age, or sex on serum CC16 was detected.[37]

Unfortunately, due to the small sample size, we could not reach solid conclusions regarding the relation between CC16 levels and aeroallergens sensitization. We found a positive correlation between cat sensitization and urine CC16 but an inverse correlation with that of cockroach and no correlation with the other allergens. Whether this is related to the type of allergen, or a reflection of different environmental exposures needs further investigation.

In conclusion, serum and urinary CC16 levels seem to be higher in atopic asthmatic children compared to healthy controls, especially during asthma exacerbation. The CC16 results in urine paralleled those in the serum which place the former as a non-invasive substitute of the latter. The potential role of urinary CC16 as a reliable biomarker that may predict pediatric asthma exacerbation and/or direct therapeutic efforts needs further wider scale studies involving timely measurements in a prospective design.

There are some limitations to our conclusions including the small sample size and lack of a non-atopic asthma control group. We did not exclude viral infections as a confounding factor especially that our study was conducted during the COVID-19 pandemic; and we could not do confirmatory tests for COVID-19 due to limited resources. Also, the consecutive manner of case enrollment led to uneven distribution of the sample according to severity and treatment modalities and this might have influenced our results.

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