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Diagnostic Value of Changes in Serum Calprotectin Level and Patients' Sputum in Response to Treatment of Cystic Fibrosis Exacerbation in Children

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Abstract

Background

Today few studies have focused on using calprotectin as an effective biomarker for monitoring the exacerbation of pulmonary complications in cystic fibrosis (CF). Thus, the present study aimed to assess the diagnostic value of the changes in the calprotectin level of patient's serum and sputum during responding to the therapy of exacerbated CF in children.

Materials and Methods: The cross-sectional study was conducted among 21 CF patients, which received required supportive and therapeutic procedures based on the protocol related to pulmonology ward in the Children Medical Research and Training Center of Tabriz University of Medical Sciences. The sputum and serum samples of all patients were collected to evaluate calprotectin level at 1-2 days after starting therapy with routine antibiotics such as cephalosporin and macrolides, and they were again gathered at the end of therapy process.

Results: Assessing outcome in 21 patients under study represented complete and partial recovery in 12 (57.2%), and 9 (42.8%) ones, respectively. The mean decrease in calprotectin level in the serum and sputum of the patients was respectively obtained as 40.72±89.08 µg/ml and 99.03±225.94 µg/ml. The calprotectin decrease in serum with the cutoff point of 15.70 µg/ml possessed the sensitivity of 66.7% and specificity of 55.6% in predicting complete recovery outcome; while that of sputum with the cutoff point of 26.20 µg/ml had the sensitivity and specificity of 66.7 and 22.2%, respectively.

Conclusion

The mean age of participants were 8.61±4.19 years. It can be concluded that serum and sputum calprotectin decrease with cutoff point of 15.70 µg/ml and 26.20 µg/ml, respectively in have high sensitivity for determining response to treatment in cystic fibrosis exacerbation.

Key Words*:* Calprotectin, Cystic fibrosis, Exacerbation, Iran, Therapeutic outcome.

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1- INTRODUCTION

Cystic fibrosis (CF) disease is considered as an autosomal recessive genetic disorder, which can affect many organs such as lungs and digestive system in children. The chronic obstruction and recurrent infections in airways, and impairment in digesting foods in bowel and its complications due to pancreatic insufficiency are regarded as the main problem in the disease (1). CF is known as the responsible for many cases of nasal polyposis, rectal prolapse, pancreatitis, gallstones, insulin-dependent hyperglycemia, failure to thrive (FTT), and liver function impairment (2, 3). Around 43-70% of neonates with CF develop bowel obstruction (4), 14.3% represent only pulmonary symptoms without digestive ones, and others reflect both symptoms with different levels (5).

The quick and timely diagnosis of the disease can be effective in decreasing CF-caused problems. The disease is classically manifested in the early childhood although 4% of patients are recognized in adulthood (6). In addition, 37-70% of patients present meconium ileus at birth and others refer with chronic respiratory complaints, FTT or both at higher age. Progressing pulmonary disease is considered as the main factor determining morbidity and mortality (7). Most patients die in the ages of 17-47 due to respiratory insufficiency (8). The most common symptoms of the disease are related to respiratory system involvement, including cough with sputum, wheezing, lung infection, and sinusitis and nasal polyps in some cases. Recurrent abdominal pain, weight loss, impairment in digesting foods, bowel obstruction, nausea and vomiting, steatorrhea, FTT, and liver involvement are the other symptoms of the disease (9, 10). Calprotectin is considered as a heterodimer protein and can bind calcium and zinc. In addition, the protein is found in neutrophil cytosol and monocyte membrane (11). Following binding monocytes to endothelium and consequently activating neutrophils, calprotectin is released, the level of which in serum or body fluids is an important index for inflammation (12). Calprotectin is used as a marker for diagnosing, responding to therapy, and monitoring in inflammatory bowel diseases (IBD) such as Crohn, ulcerative colitis, bowel polyps, and colorectal cancer (13, 14). An increase in fecal calprotectin is proposed as a reliable method for proving IBD diagnosis, differentiating from irritable bowel syndrome (IBS), and monitoring its activity after recognition (15).

Fecal calprotectin is considered as a new screening test which indicates the activity of fecal leukocytes. Due to the stability of calprotectin in fecal samples at room temperature for 10 days, patient can collect his sample in the home (16). Numerous studies have focused on calprotectin as a marker for recognizing, responding to therapy, and monitoring in IBD (16, 17). However, few studies highlighted applying calprotectin as an efficient biomarker in monitoring the exacerbation of pulmonary complications in CF (18). Thus, the present study aimed to evaluate the diagnostic value of the changes in the serum and sputum calprotectin of patients in responding to the therapy of exacerbated CF.

2- MATERIALS AND METHODS

2-1. Design and duration of the study

The present cross-sectional study was conducted in the Pediatric Health Research Center belonged to the Children Medical Research and Training Center of Tabriz University of Medical Sciences during 18 months from March 2019 to August 2020. Exacerbated CF patients with age under 14 years old were selected. All of the selected patients received supportive, care, and therapeutic procedures by considering the diagnosis. The parents of patients in both

groups completed written consent for participating their children in the study. Additionally, the study was approved by ethics committee in the Tabriz University of Medical Sciences with the code of IR.TBZMED.REC.1398.619.

2-2. Population and inclusion criteria

Due to the limited number of the children hospitalized with exacerbated CF diagnosis during one year and conduction of a relatively comprehensive study, all exacerbated CF patients referred during 2019 were included in the study through convenience sampling in order to determine the sample size. Finally, 21 patients were selected. The inclusion criterion was confirmed CF patients who had recently the shortness of breath, as well as wheezing and increasing sputum excretion. Concurrent and acute pulmonary and digestive developing other pulmonary disease, along with consuming antibiotics before starting therapeutic criteria. involvement, being in urgent status, CF-caused pulmonary problems, and process were considered as exclusion.

2-3. Method

All patients were visited by a pediatric pulmonologist and evaluated with respect to inclusion and exclusion criteria before entering the study. Attending physician confirmed the exacerbation of pulmonary symptoms in the patients based on the recent shortness of breath, as well as recent wheezing and increasing sputum expulsion. Before entering the patients into the study, the objective and method of its implementation were explained to their parents. Then, the patients included in the study after getting written informed consent from the parents. The required supportive and therapeutic procedures were implemented in all patients based on the protocol of pulmonology ward in the Children Medical Research and Training Center of Tabriz University of Medical Sciences. Additionally, they received no additional therapeutic and intervention procedure. The sputum and serum samples of all patients were collected at 1-2 days after starting therapy with routine antibiotics such as cephalosporin and macrolides, as well as the end of therapy process in order to assess calprotectin level. Further, the therapy process of patients, which was lasted 1-3 weeks, was followed by evaluating their clinical symptoms under the supervision of a pediatric pulmonologist.

Sputum was processed within 2 hours of collection. Sputum plugs were harvested and processed with 4x weight/volume 0.1% dithiothreitol (DTT) after which 4x weight/volume PBS was added. Samples were filtered through 48 μm mesh and centrifuged at 1200 rpm to remove the cells. Supernatant was stored at -80 °C until further analysis. The cell pellet was re-suspended in PBS, cytospins prepared and stained with May-Grunwald-Giemsa for differential cell counting. All counts were expressed as percentage of the population counted. All samples utilized in the study contained p40% squamous cells ensuring samples were from the lower airway (6).

Blood was collected into serum tubes with pre-added clotting activator (Monovette serum collection tubes, Sarstedt AG and Co, Germany). The tube was then mixed by inverting five times. Blood was left to clot at room temperature for 45 minutes. Tubes were centrifuged at 1800 × g for 15 minutes at room temperature. Separated serum was removed into cryovials (Nunc, Thermo Fisher Scientific, Denmark) as above and stored at -80 °C until further analysis. A separate EDTA blood sample was taken for routine hematology (white cell count) (6). Calprotectin was measured in sputum and serum by a double antibody sandwich ELISA, using monoclonal and polyclonal antibodies against human calprotectin complex (gift of Erling

Sundrehagen, Norway). Standard curve and dilutions for sputum ELISAs were performed in the presence of 0.05% DTT to ensure accurate measurement of mediators in sputum as samples had been processed with DDT (6). CRP values were measured at 1-2 days of antibiotic therapy and end of 1-3 week therapeutic process, along with determining sputum and serum calprotectin in order to compare their diagnostic value. Further, monitoring clinical symptoms was considered as recovery criterion in patients. All of the items under study were recorded in the relevant checklist and data were initially collected.

2-4. Statistical analysis

Demographic data analyzed by descriptive statistics such as frequency, percentage, and mean±standard deviation (SD). Mann-Whitney U test used for comparing calprotectin values in before and after treatment times. Relation between serum and sputum calprotectin level was evaluated by Spearman's correlation. All data analyzed by using SPSS 18 software, and p-value less than 0.05 was considered as statistically significant. Diagnostic value (sensitivity and specificity) of test were analyzed by ROC Analysis using ROC Curve with cut-off point.

3- RESULTS

The cross-sectional study was conducted among 21 children with CF exacerbation referred to the Children Medical Research and Training Center in Tabriz during 2019. Patient's demographic data were in **Table.1**.

Table-1: Patient's demographic data.

Factor	Result
Age	
Mean ± SD	8.61±4.19 years
Median	10 years
Minimum	6 months
Maximum	14 years
Boys (Mean ± SD)	10.38±2.90 years
Girls (Mean ± SD)	5.75±4.42 years
Gender	
Boy	13 (61.9%)
Girl	8 (38.1%)

SD: Standard deviation.

The most common initial clinical symptom of patients was related to cough with sputum excretion with the frequency of nine (42.9%) (**Figure.1**). Further, the maximum frequency was observed in the therapeutic regime of amikacin-ceftazidime (76.2%). Further, the maximum frequency was observed in the therapeutic regime of amikacin-ceftazidime (76.2%). Mean of treatment time in patients was 12.09±4.63 days (median=14 days; range=3 to 27 days). The mean of therapy duration in boy and girl patients were respectively 12.53±5.48 and 11.37±2.97 days by representing no statistically significant difference (p=0.537). Based on evaluating outcome in 21 patients, 12 (57.2%) and nine (42.8%) ones were respectively recovered completely and partially, and the morality was zero. Regarding serum, initial and final calprotectin were measured in the patients, the mean of which was 253.14± 534.89 (median=103.60), and 212.39±

462.92 (median=74.20), respectively. In addition, the minimum and maximum of initial calprotectin were obtained as 5.50 and 2320, respectively; while those of final one were 7.00 and 2027, respectively. The normal range of serum calprotectin in stable cystic fibrosis patients were 16.4 to 93.9 (µg/ml) (19). The mean of calprotectin decrease in serum was determined as 40.72±89.08, the comparison of which with the outcome of patients demonstrated insignificant difference between complete and partial recovery (p=0.686) (**Table.2**).

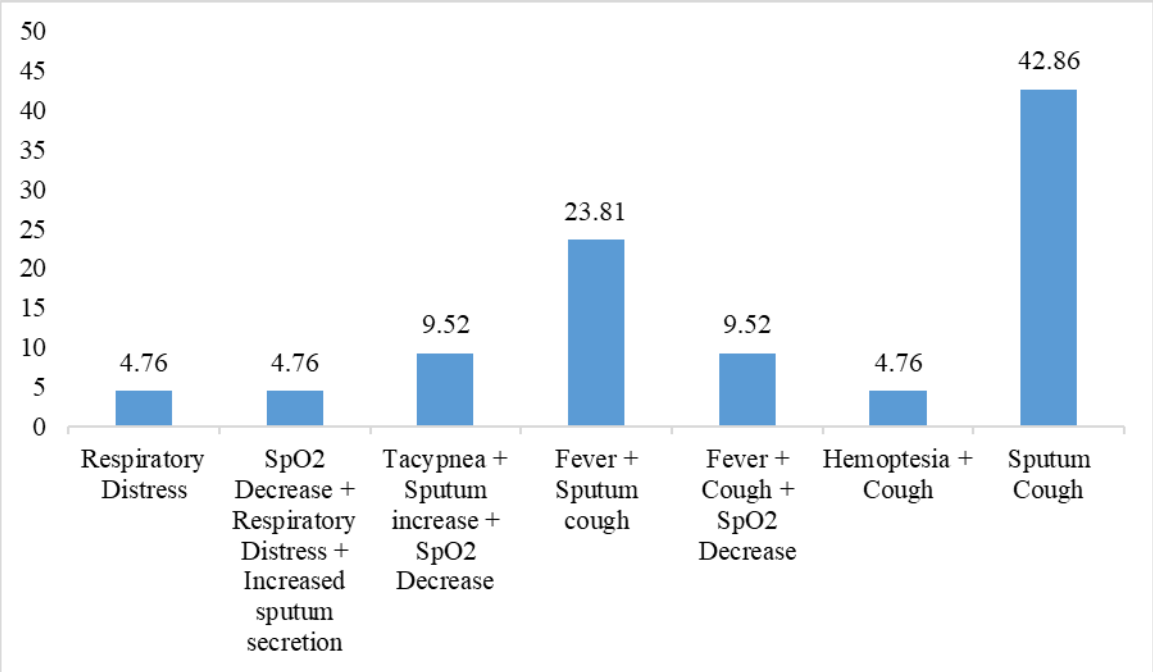

Fig. 1: Frequency of initial clinical symptoms in the patients.

The above-mentioned measurements were repeated in sputum, by indicating the mean of initial and final calprotectin as 806.15±2199.48 (median=116.00) and 707.12±2043.31 (median=57.60), respectively. Additionally, the maximum and minimum of initial calprotectin were respectively achieved as 7426 and 23.20, while those of final one were 7323 and 13.80, respectively. The normal range of serum calprotectin in stable cystic fibrosis patients were 32 to 157 (µg/ml) (20). Further, the mean reduction of sputum calprotectin was obtained as 99.03±225.94, the comparison of which with the outcome in patients represented no significant difference between complete and partial recovery (p=0.368) (**Table.2**). Furthermore, calprotectin decrease in serum was significantly correlated to that of sputum based on the Spearman's test (p=0.011, r_p=0.545).

Table-2: Comparing the mean decrease in serum and sputum calprotectin based on the outcome.

Specimen	Final Outcome	Mean ± SD	P-value
Serum (µg/ml)	Complete Remission	48.08±81.25	0.686
	Partial Remission	30.97±102.83	
Sputum (µg/ml)	Complete Remission	52.15±46.16	0.368
	Partial Remission	161.53±342.06	
* In this table the mean ± SD reduction of serum and sputum calprotectin were indicated.			

The diagnostic value of response to therapy was examined through ROC curve analysis based on the final values and calprotectin reduction in the patients (**Figure.2**). In this regard, obtaining complete recovery was considered as response to therapy (**Table.3**).

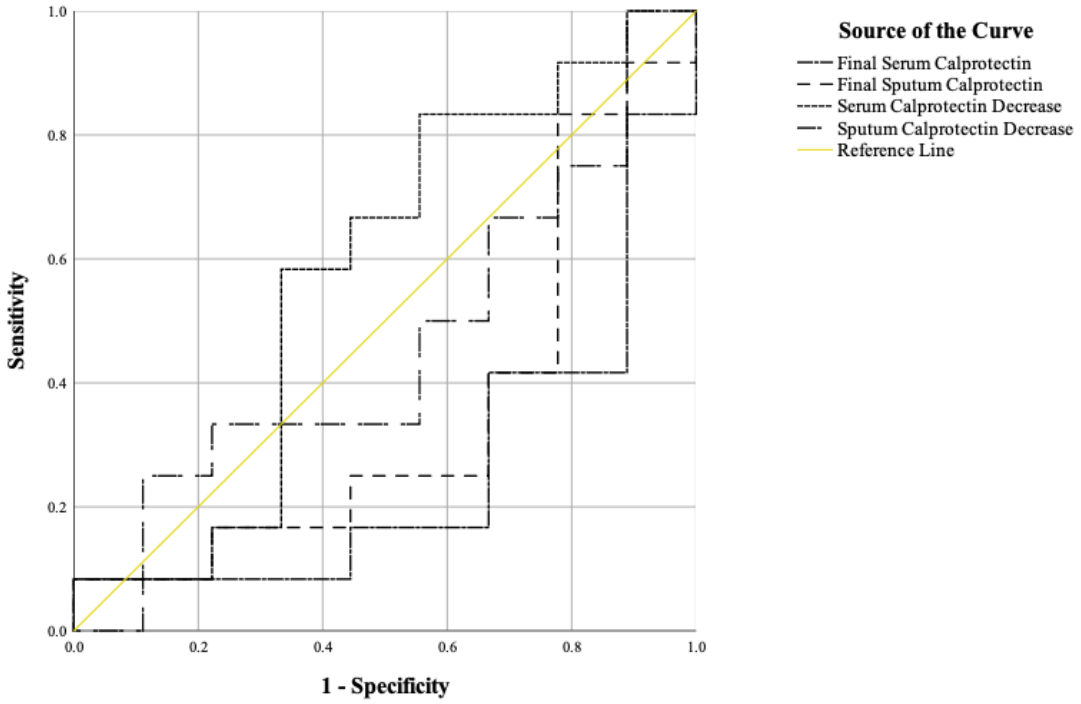

Fig. 2: ROC curve for comparing the changes in calprotectin in the patients under study.

Table-3: Diagnostic value of response to therapy based on the final values and calprotectin decrease in serum and sputum samples.

Parameters	Final Serum Calprotectin	Final Sputum Calprotectin	Serum Calprotectin Decrease	Sputum Calprotectin Decrease
Area under curve	0.259	0.352	0.574	0.463
Standard Error	0.115	0.129	0.136	0.132
p-value	0.065	0.256	0.047	0.029
Lower Bond	0.034	0.099	0.307	0.205
Upper Bond	0.484	0.605	0.841	0.721
Cut off	32.15	35.45	15.70	26.20
Sensitivity (%)	75	58.3	66.7	66.7
Specificity (%)	39	32.2	55.6	22.2

Assessing initial CRP level in the patients represented 10 (47.6%), 6 (28.6%), and 5 cases (23.8%) as negative, +1, and +2, respectively. However, 13 (61.9%), 4 (19%), and 4 (19%) cases of final CRP level were respectively determined as negative, +1, and +2 at the end of therapeutic process (**Table.4**).

Table-4: Comparing initial and final CRP in the patients under study.

Case No.	Primary CRP	Final CRP	Final Outcome
1	+1	Negative	Partial Remission
2	Negative	+2	Partial Remission
3	+2	+1	Partial Remission
4	Negative	Negative	Complete Remission
5	+1	Negative	Complete Remission
6	Negative	Negative	Complete Remission
7	Negative	Negative	Partial Remission
8	Negative	Negative	Complete Remission
9	Negative	Negative	Complete Remission
10	+2	+2	Partial Remission
11	Negative	Negative	Complete Remission
12	+1	+2	Complete Remission
13	+2	+2	Partial Remission
14	Negative	Negative	Partial Remission
15	+2	+1	Partial Remission
16	+1	Negative	Complete Remission
17	+2	+1	Partial Remission
18	Negative	Negative	Complete Remission
19	+1	+1	Complete Remission
20	Negative	Negative	Complete Remission
21	+1	Negative	Complete Remission

* CRP: In CF patients CRP evaluated by qualitative method as negative, +1 and +2. CRP was evaluated first before treatment and then after treatment. Then, for each patient, the differences were demonstrated.

4- DISCUSSION

The present study was conducted among 21 children with exacerbated CF. In this regard, the calprotectin level in serum and sputum samples were measured at the beginning of the study and end of therapeutic process. The patients under study were averagely aged as 8.61±4.15 years, among whom 61.9% were boy. In addition, the age of boy patients was more significantly (p=0.024). Further, sputum-associated cough was determined as the most common clinical symptom observed at the beginning of referral (42.9%). Furthermore, the mean of therapy duration was obtained as 12.09±4.63 days, and no difference was observed between two genders in this regard (p=0.537). Based on evaluating outcome in 21 patients, 12 (57.2%), and nine (42.8%) ones were completely and partially recovered, respectively. Additionally, the morality in the patients was zero. Considering the comparison of the mean decrease in serum calprotectin (40.72±89.08), as well as that of sputum one (99.03±225.94) with outcome in the patients, there was an insignificant difference between complete and partial recovery (p=0.686 and 0.368, respectively). Further, a direct and

significant correlation was observed between the calprotectin reduction in serum and sputum based on the Pearson's test (p=0.011, r=0.545). Raats et al. evaluated serum and sputum calprotectin in bronchiectasis disease monitoring. In their study the mean sputum calprotectin was 51.1±28.8 µg/ml during exacerbation and 38.8±27.8 µg/ml in stable condition (p=0.086). This result were in concordance with our study (29). Based on the results of assessing the diagnostic value of calprotectin in response to therapy, the sensitivity and specificity of decreasing serum calprotectin (cut-off point =15.70) in predicting complete recovery outcome were 66.7% and 55.6%, respectively, while those of sputum (cut-off point = 26.20) were 66.7 and 22.2%, respectively. The diagnostic value of changes in the CRP related to the patients was less, compared to that of calprotectin.

Gray et al. found that calprotectin decreases in exacerbated CF patients by implementing antibiotic therapy (6), which is consistent with the results of the present study. Some researchers reported the higher level of calgranulin A and B, as the subunits of calprotectin, in the sputum and bronchoalveolar lavage fluid (BALF) samples of CF patients (6, 21). Rumman et al. studied the associations of elevated fecal calprotectin among CF patients and whether its level correlates with the clinical manifestations of CF. Results showed that were no significant differences between CF patients with normal and abnormal fecal calprotectin levels. However, patients who were not receiving inhaled antibiotics had higher fecal calprotectin levels than those who were. They concluded that Elevated fecal calprotectin might not accurately predict intestinal inflammation in CF. However, the fact that it was elevated in both pancreatic sufficient and insufficient groups supports the concept of “cystic fibrosis enteropathy” regardless of the pancreatic status (22). In the study of Reid et al. they evaluated measurement of serum calprotectin in stable patients predicts exacerbation and lung function decline in cystic fibrosis. They reported single measurement of serum calprotectin in stability predicts changes in disease activity manifest by time to next exacerbation and decline in lung function. Measurement of calprotectin in the clinic may allow the identification of patients at high risk of exacerbation and/or lung function decline and allow appropriate tailoring of therapy (19). Calprotectin can be secreted from activated neutrophils (23), released after cell death (24), or considered as an appropriate marker for airway inflammation in CF patients. In addition, fecal calprotectin can be regarded as a good marker for diagnosing organic bowel disease (25), and used for differentiating IBD in which neutrophils are dominant compared to IBS (26).

Further, calprotectin can play an important functional role in the airways of CF patients, the role of which was evaluated through the animal models of pulmonary diseases in the recent studies (27). Furthermore, the direct inhibition of calprotectin in pneumonia mice models results in decreasing the migration of inflammatory cells, which indicates the direct role of the marker in migrating the cells (27). Considering the above-mentioned results, the changes in sputum calprotectin following antibiotic therapy in CF patients indicate the positive relationship between calprotectin with the variations in the airway inflammatory status of the patients. Cellular and molecular studies should be conducted in future to assess the main role of calprotectin as a pro-inflammatory molecule in lung. In the present study, the calprotectin decrease in sputum after antibiotic therapy was more than that of serum. In addition, serum calprotectin increased in four patients, while the trend

was not observed in sputum samples, which demonstrates lower incorrect changes in sputum calprotectin. In the study of Jung et al. reported that CRP and calprotectin could discriminate stable vs. pulmonary exacerbation visits with good performance and appear promising as diagnostic biomarkers but further validation studies are required prior to implementing these diagnostic thresholds (28). Further, the calprotectin level of serum was nearly four times less than that of sputum sample, by representing the high concentration of calprotectin in the sputum sample of the patients.

An increase in sputum calprotectin occurs locally and is caused by neutrophils, especially the necrotic ones abundant in the sputum of CF patients with negative-gram infections (30). Furthermore, the variations in serum calprotectin can be related to an increase in neutrophil migration from bone marrow or leakage of calprotectin from lungs into blood flow because of destruction lung epithelium severely, which necessitates further studies. Also, our study hadn't control group. Regarding the present study, CRP reduction was only observed in four patients and no difference was obtained between initial CRP and that after therapy for others. However, the study possessed several limitations such as low sample size and qualitative CRP measurement, leading to measurement bias. Gray et al. reported a significant decrease in CRP level after therapy (6).

5- CONCLUSION

Based on the results of the present study, the calprotectin level in the serum and sputum of the exacerbated CF patients reduced significantly after antibiotic therapy, and possessed higher diagnostic value compared to CRP does. Significantly, serum calprotectin decrease has sensitivity and specificity of 66.7% and 55.6%, respectively with a cutoff point of 15.70 in prediction of CF exacerbation. In addition, significantly sputum calprotectin decrease has sensitivity and specificity of 66.7% and 22.2%, respectively with a cutoff point of 26.20.

6- ACKNOWLEDGMENTS

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7- CONFLICT OF INTEREST: None.

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