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Title: Surfactant Protein D, a clinical biomarker for COPD with excellent discriminant values

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Running title: AKIKI et al: SP-D, a clinical biomarker for COPD with excellent discriminant values

Abstract

Biological markers can help to better identify a disease or refine its diagnosis. We studied the association between Surfactant Protein D (SP-D) and Chronic Obstructive Pulmonary Disease (COPD) among subjects consulting for respiratory diseases or symptoms and we compared it to C-reactive protein (CRP) and Fibrinogen. We aimed as well to find the optimal cut-off point of SP-D able to discriminate COPD patients. A case-control study including 90 COPD patients, 124 asthma patients and 180 controls was conducted. Standardized questionnaires were administered and lung function tests were performed. Biological markers were measured in blood according to standardized procedures. Association between SP-D and COPD was investigated using logistic regression models. Receiver-operating curves were used for threshold finding. SP-D levels above the median value were positively associated with COPD (adjusted OR=3.86, 95%CI: 1.51-9.85, p=0.005). No associations were found between CRP, fibrinogen levels and COPD or asthma. Scores for COPD diagnosis including all COPD patients or ever smokers COPD patients were identified (sensitivity of 76.4% and 77.8%, specificity of 89.3% and 88.5% respectively). SP-D seems to be able to differentiate COPD from patients consulting for other respiratory symptoms or diseases. Used with socio-demographic characteristics and respiratory symptoms, SP-D could discriminate COPD patients from controls particularly among smokers.

Introduction

Chronic obstructive pulmonary disease (COPD) is ranked as the fourth leading cause of death worldwide [1]. A diagnosis of COPD is considered in any patient who has dyspnea, chronic cough or sputum production, a history of exposure to the risk factors, but still needs a confirmation by spirometry [1]. This technique is however difficult for the patient, not always available, requires a certain expertise for its interpretation and is poorly correlated to disease severity [2,3]. As an alternative, research has focused recently on the measurement of a variety of biomarkers that seem to exhibit prospective utility in the diagnosis and prognosis of COPD. Serum levels of systemic markers of inflammation such as C-reactive protein (CRP) and fibrinogen, which are validated in the clinical laboratory assays [4], have been shown to reflect

the degree of severity of airway inflammation [5,6,7] but they are not lung-specific [7-10]. In addition, there is no approved predictive or prognostic biomarker for COPD reported to date.

Recent studies have described associations between a pulmonary-specific marker, surfactant protein D (SP-D) and COPD [11,12]. SP-D is a large hydrophilic glycoprotein that belongs to the collectin family, mainly produced in the lung by alveolar type II cells and non-ciliated Clara cells [13]. SP-D facilitates the resolution of lung inflammation [14], is detectable in serum, and has the advantage to be a stable molecule over a period of 6 months [15].

In our study, we hypothesized that SP-D is a more specific biological marker than CRP or fibrinogen to differentiate COPD patients among individuals consulting for respiratory diseases or symptoms including those with asthma. So, we studied the associations between SP-D, CRP or fibrinogen and patients' COPD or asthma and controls. Then, we searched for the optimal cut-off point able to discriminate COPD patients from controls using serum SP-D levels.

Methods

Study design and subjects

All COPD patients consulting at the outpatients' clinics of the Pulmonology department of a university hospital and medical center in Beirut between June 2011 and April 2013 were recruited in this case-control study. COPD diagnosis was defined as having a post bronchodilator ratio of the forced expiratory volume at one second (FEV_1)/forced vital capacity (FVC) being less than 70% [1]. Ninety COPD patients were classified into categories based on GOLD guidelines 2013: 35 in group A, 38 in group B, 3 in group C, and 14 in group D. None of the patients had an exacerbation one month prior to the inclusion. Considering medication, COPD patients were classified as treated when they were receiving beta2-agonists or anti-cholinergics (whether short acting or long acting) combined or not with inhaled corticosteroids (ICS), as suggested previously [16]. A total of 46 COPD patients were found to be on regular medication. Patients with asthma (n=124) were defined according to GINA guidelines 2012. A clinical diagnosis based on patients symptoms (breathlessness, wheezing, cough and chest tightness), family history of asthma, worsening symptoms when exposed to various risk factors was performed and lung function measurements were assessed [17]. Having another respiratory disease was the exclusion criterion for COPD or asthma patients. Healthy individuals from the

general population (n=92) and outpatients consulting for a variety of non-respiratory problems in the same hospital (n=88) were recruited. Controls had normal lung function tests as defined by GOLD guidelines [1]. The exclusion criteria were previous or current diagnosis of any respiratory disease such as asthma, COPD, chronic bronchitis, fibrosis, tuberculosis or lung cancer. Written informed consent was signed by all participants.

Questionnaire

A standardized questionnaire, adapted to the local Arabic language, was filled out by all participants with the help of trained interviewers. It included information on the socio-demographic characteristics, exposure to the disease risk factors, clinical assessment of the disease and its symptoms (American Thoracic Society Questionnaire [18], the Medical Research Council "MRC" breathlessness scale [19]), and smoking history.

Blood collection and pulmonary function tests

Before pulmonary function tests, venous blood samples were withdrawn from all participants, immediately transported at 4°C to the Immunology Laboratory of the Lebanese University and centrifuged as well at 4°C within 12 hours after withdrawal. Plasma and serum were then aliquoted and stored frozen at -20°c until use within 6 months.

All participants underwent baseline spirometry by a trained physician. Reversibility assessments (post-bronchodilator spirometry) were performed after inhaling two puffs of Ventoline with 30 minutes delay from the baseline spirometry.

Measurement of SP-D, CRP and Fibrinogen

Quantification of SP-D was performed by a five-layered ELISA as previously described by Leth-Larsen et al. [20]. Briefly, microtiter plates were coated with $F(ab')_2$ anti-human SP-D in sodium carbonate buffer. After overnight incubation at 4°C, the plates were washed and left with washing buffer (TBS, 0.05% Tween20, 5mM CaCl2) for 1 hour at room temperature. Calibrator, controls and samples were added at the appropriate dilution and incubated overnight at 4°C. This was followed by successive incubations with biotinylated monoclonal anti-human SP-D antibody, horseradish peroxidase-conjugated streptavidin and O-phenylenediamine in citrate-phosphate buffer pH 5, containing 0.014 % H_2O_2. Plates were read at 492 nm in a multichannel

spectrophotometer after adding H_2SO_4. Serum CRP and plasma Fibrinogen were measured using double antibody sandwich ELISA kits (Immunology Consultants Laboratory, Inc. USA) according to the manufacturer's instructions. All samples were tested in duplicate. The coefficient of variation was 4.8% for SP-D, 2.7% for CRP, and 2% for Fibrinogen.

Statistical analyses

Fibrinogen was normally distributed, whereas SP-D and CRP levels were not even after log transformation. Results are expressed as median (interquartile ranges). Differences between groups were tested using the t-test or Chi-squared when appropriate (non-parametric tests gave same results). Associations between biomarkers and lung function tests were estimated using general linear models with adjustment for age, sex, body mass index (BMI) classes and smoking status. Three logistic regressions models were used to evaluate the association between SP-D and COPD. In the first and second regressions, COPD patients *vs.* controls were used as dependent variable. The independent variables were SP-D expressed as above/below the median, and all potential confounding variables such as age, gender, BMI classes, and smoking, and all the remaining socio-demographic characteristics and respiratory symptoms (cough, wheeze and expectoration) having $p<0.2$ in the bivariate analysis. As a sensitivity analysis, the second regression was performed in ever smokers only (smokers and ex-smokers). Then, in order to confirm the availability of SP-D to differentiate COPD from asthma patients, a third regression including COPD *vs.* asthma patients as dependent variable was performed. The adjusted odds ratios (aOR) obtained from the first and second regressions respectively were then rounded to the nearest units and used as coefficients in score 1 (for all COPD patients and controls) and score 2 (for ever smokers COPD patients and controls) with the purpose to predict COPD diagnosis. Receiver-operating curves were then generated to determine the ability of the scores 1 and 2 to discriminate between COPD patients and controls. The level of statistical significance was set at < 0.05. All analyses were performed using SPSS 17.0.

Based on a previous study on SP-D in COPD [21], the smallest difference that could exist between healthy individuals and COPD patients was of 56.6 ng/mL, while the standard deviation was of 80.2 ng/mL. With an alpha error of 5%, a power of 80%, a minimum of 32 patients and 46 controls was required for the study.

Results

Participants' characteristics

Characteristics of patients and controls are described in table 1. The three groups were significantly different regarding age, sex, smoking, marital status and education. Compared to controls, patients with COPD were more frequently men, ever smokers and unmarried (all p-values ≤ 0.003); while patients with asthma were younger, more often unmarried, non-smokers and had more university degrees (all p-values ≤ 0.02). Compared to asthma patients, COPD patients were older, more frequently men, ever smokers and had less university degrees (all p-values ≤ 0.001). No other significant associations were found.

Association between biological markers and COPD and asthma

The three groups were significantly different regarding SP-D levels only. Serum SP-D levels were significantly increased in COPD patients as compared to controls and to asthma patients. There were no significant differences in SP-D levels between asthma patients and controls. No other significant associations were observed (table 2).

SP-D levels were lower in COPD patients that were on inhaled therapy (ICS and/or bronchodilators) as compared to those not but the difference did not reach the level of significance [n=46 *vs.* 42, 1507 ± 868 *vs.* 1830 ± 971 $ng.mL^{-1}$ respectively, p=0.1].

There were no significant associations between biological markers and FEV1% predicted or FEV1/FVC after administration of a bronchodilator in COPD patients (data not shown).

Association between SP-D and COPD - Multivariate analyses

Multivariate analyses performed on all COPD patients and controls showed that SP-D levels above the median value, male gender, being unmarried and symptoms such as having a morning cough, cough during the day and wheeze during the day were significantly and positively associated with COPD (table 3a). Same results were observed in ever smokers COPD patients and controls (table 3b).

In the third regression (table3c) having COPD patients *vs.* asthma patients as dependent variable, SP-D levels above the median value, older age, male gender and having a morning cough were significantly and positively associated with COPD.

Score for COPD diagnosis: construction, properties and thresholds

Taking into account the adjusted OR from the first regression (from table 3a) and rounding the nearest unit, a first score for COPD diagnosis was computed.

Score1 = (SP-D above/below the median*4) + (gender *3) + (marital status*3) + (cough in the morning*39) + (cough during the day*11) + (wheeze during the day*65) + (smoking*1).

In COPD patients, score 1 has a minimum of six and a maximum of 129. The mean was 47.2, the median 49 and the standard deviation of 31.9. In controls, the minimum was six; the maximum was 75 with a mean of 12.6, a median of 10 and a standard deviation of 10.1.

Receiver-operating characteristic curve generated for score 1 is shown in Figure 1, comparing COPD patients to controls. The area under the curve was at 0.890 (0.841-0.940; $p<0.001$). The most optimal cut-off point was 15.5 (table 4) at which point sensitivity, specificity, positive predictive value and negative predictive value were 76.4%, 89.3%, 81% and 74% respectively.

Figure 1: Receiver-operating characteristic curve (ROC) with all COPD patients and controls.

A second score (score 2) was calculated from the second regression (from table 3b) for ever smokers COPD patients and controls.

Score 2 = (SP-D above/below the median*6) + (gender *4) + (marital status*3) + (cough in the morning*54) + (cough during the day*9) + (wheeze during the day*36).

In ever smokers COPD patients, score 2 has a minimum of 10 and a maximum of 116. The mean was 47.7, the median 51 and the standard deviation of 28.5. In ever smokers' controls, the minimum was 7; the maximum was 67 with a mean of 15, a median of 13 and a standard deviation of 11.4.

A receiver-operating characteristic curve was generated from score 2 (Figure 2). The area under the curve was at 0.895 (0.841-0.950; $p<0.001$). The most optimal cut-off point was 18.5 at which point sensitivity, specificity, positive predictive value and negative predictive value were 77.8%, 88.5%, 70% and 82% respectively (table 5).

Figure 2: Receiver-operating characteristic curve (ROC) with ever smokers COPD patients and controls.

Discussion

In this study, we investigated the association between serum SP-D and COPD among subjects consulting for respiratory diseases or symptoms, and in comparison with serum CRP and plasma Fibrinogen. SP-D levels were significantly and positively associated with COPD while serum CRP and plasma fibrinogen levels were not. Furthermore, we found a score for COPD diagnosis with excellent discriminant values, the best scale for diagnosing COPD being obtained using SP-D levels, socio-demographic characteristics, smoking status and respiratory symptoms significantly associated with COPD.

The particular selection of patients and controls allowed us to support our hypothesis that SP-D is able to differentiate COPD patients among individuals consulting for respiratory diseases or symptoms, including those with asthma. We recruited COPD and asthma patients and controls' healthy and outpatients consulting for non-respiratory diseases. Regarding the study limitations, we assessed the effect of ICS on SP-D levels by regrouping patients receiving beta2-agonists (or anticholinergics) combined or not with ICS due to sample size and to the percentage of COPD patients in severe and very severe stages, thus requiring treatment with corticosteroids. SP-D levels were lower in the group of COPD patients that were receiving treatment *vs.* not but the association did not reach the level of significance as described previously [12,22,23]. We might have a selection bias (75.8% of COPD patients were ever smokers *vs.* 62.2% of controls) but it did not affect our analytical results. Recall bias might be possible because information on previous smoking history were based on self-report. The information bias introduced by underreporting is probable, as smoking behaviors are sensitive issues, especially among patients that don't want to quit smoking. However, as patients knew that this study outcome may be beneficial in their medical follow up, we assumed that the information bias is minimized.

We found that SP-D levels were significantly and positively associated with COPD as compared to controls, result in line with previous studies [11,12,24]. To our knowledge, our study is the first to confirm such result through multivariate analyses. The association between SP-D and COPD was previously investigated by bivariate analyses [11,24,25,26]. Ou et al. have did not found an association between SP-D and COPD in the bivariate and multivariate analyses [27]

and Illumets et al. have adjusted over a single confounding factor which is age [21]. Others have performed multivariate analyses for the association of SP-D but considering COPD exacerbation rather than diagnosis [12,22,27]. Since smoking is known to highly influence SP-D levels, we also considered ever smokers only, and we found that SP-D remained significantly and positively associated with COPD, result concordant with previous literature [11,12,28]. We found no association between CRP or Fibrinogen and COPD. Ju et al. previously reported no association between CRP and stable COPD patients [24]. While others studies have reported higher CRP or fibrinogen levels in stable COPD patients compared to controls [29,30,31]. However, the use of CRP and Fibrinogen as biomarkers for COPD is limited by their low specificity or low predictive value for COPD [16] and any inflammatory or infectious even if not related to lung inflammation can modify CRP levels [32]. In our study, SP-D levels were also significantly elevated in COPD patients as compared to asthma patients, result in agreement with those of Mutti et al. [26]. We observed no significant differences in SP-D levels between asthma patients and controls. In fact, there is no clear evidence regarding the association between SP-D and asthma. Some have reported no association of SP-D levels with asthma [26] while a variety of small clinical/case studies have indicated raised serum SP-D levels associated with allergy [29,33]. Finally, SP-D seems to be a clinical biomarker for COPD, able to differentiate COPD patients among individuals consulting for respiratory diseases or symptoms including those with asthma, while serum CRP and plasma fibrinogen levels did not.

We did not find an association between SP-D and FEV1% predicted or FEV1/FVC in COPD patients. Previous studies did not report clear evidence of association between biomarkers levels and lung function parameters. Some of them have reported similar results whether in the bivariate analyses [12,34,35] or in the multivariate analysis [36]. However, some have described significant negative correlations between SP-D levels and lung functions in bivariate analyses with a borderline p-value for the association between SP-D and FEV1% predicted [37] or a weak but significant association with FEV1/FVC in only 20 COPD smokers [11]. Others have found an association with FEV1 through multivariate analyses in only 23 patients with advanced COPD [38], in severe COPD patients [24] or in smokers only [39]. Regarding CRP and Fibrinogen, our results are also concordant with previous studies who found no association with FEV1% in COPD patients [38,40]. On the contrary, others have found an inverse relation

between CRP or Fibrinogen and lung function tests [7,36]. Overall, differences between the studies may be explained in part by difference in the sample size, inclusion or exclusion of non-smoker COPD patients and severity of the disease. In our study, eighty one % of the COPD patients were classified in group A and B and nineteen % in groups C and D and we included smokers, ex-smokers and non-smokers COPD patients.

To our knowledge, our study is the first to suggest a score for COPD diagnosis with excellent discriminant values and validity results (area under the curve, sensitivity, specificity, positive predictive value and negative predictive value). There are validated diagnosis scales that could be used in primary care settings without blood measurements such as DS-COPD (diagnosis score for COPD patients) [41]. A previous study has carried out ROC curves on 44 stable COPD patients to evaluate diagnosis accuracy of SP-D. They found a total area under the curve of 0.734 [21]. To get more accurate results, we generated ROC curves for 2 scores calculated from the variables associated with COPD in the logistic regression models for all COPD patients and controls and for ever smokers COPD patients and controls respectively and including in addition to SP-D, the socio-demographic characteristics and the respiratory symptoms retained by the model and associated with COPD. We had an area under the curve of 0.890 and of 0.894 among all subjects and ever smokers respectively, which may be considered as improved results compared to previous literature.

In conclusion, SP-D seems to be able to differentiate COPD from patients consulting for respiratory symptoms or diseases. Used along with socio-demographic characteristics and respiratory symptoms associated with COPD, SP-D could discriminate COPD patients from controls particularly among smokers.

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Conflict of interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Table 1: Characteristics of controls, COPD and asthma patients.

	Controls (N=180)	COPD patients (N=90)	Asthma patients (N=124)
Age	55 (51;64)	62 (50;71)	46 (32;61)
BMI classes[a]			
Normal	52 (29.9)	33 (37.1)	46 (37.4)
Overweight	82 (47.1)	33 (37.1)	44 (35.8)
Obese	40 (23.0)	23 (25.8)	33 (26.8)
Gender, (Male %)	65 (36.5)	52 (57.8)	39 (32.2)
Marital status			
Married	134 (84.3)	61 (71.8)	70 (60.9)
Education			
University level	28 (16.4)	12 (13.8)	43 (35.0)
Work (yes %)	69 (39.9)	35 (41.7)	55 (45.8)
Ever smokers	112 (62.2)	68 (75.6)	60 (48.4)

Abbreviations: COPD, Chronic Obstructive Pulmonary disease; BMI, Body Mass Index; Q, quartile; %, percentage.

[a]BMI classes according to the World Health Organization's classification.

Age expressed as Median (Q1-Q3); All others as n (%).

Table 2: Variation of SP-D, CRP and Fibrinogen levels between patients and controls

	Controls (N=180)	**COPD patients (N=90)**	**COPD patients compared to controls**	**Asthma patients (N=124)**	**Asthma patients compared to controls**	**COPD patients compared to asthma patients**
SP-D	1269	1510	**0.02**	1130	0.7	**0.02**
(ng/mL)	(664;1884)	(986;2174)		(676;1852)		
CRP	9.72	8.41	0.5	8.35	0.4	0.3
(ng/mL)	(4.37;15.5)	(3.48;14.3)		(3.35;15.8)		
Fibrinogen	3135	2992	0.5	3358	0.09	0.06
(µg/mL)	(2730;3597)	(2550;3965)		(2575;4079)		

Abbreviations: COPD, Chronic Obstructive Pulmonary Disease; SP-D, Surfactant Protein-D; CRP, C-reactive protein; Q, Quartile.

Results expressed as Median (Q1-Q3).

Table 3: Association between serum SP-D levels and COPD - Multivariate analyses

(a) - First regression – N =221 – COPD patients vs. controls as dependent variable			
	aOR	**95% CI**	**P-value**
SP-D (above the median)	3.86	1.51; 9.85	0.005
Male gender	2.92	1.23; 6.93	0.02
Not Married	3.07	1.19; 7.96	0.02
Cough in the morning	39.2	10.1; 153	<0.001
Cough during the day	10.9	3.25; 36.6	<0.001
Wheeze during the day	64.9	11.8; 356	<0.001
Ever smokers	1.31	0.54; 3.18	0.5
Nagelkereke R^2= 60%; Hosmer & Lemeshow = 0.541; 86.4% of the subjects correctly classified.			
(b) - Second regression – N =141 – COPD patients vs. controls as dependent variable for ever smokers			
SP-D (above the median)	6.26	1.81; 21.65	0.004
Male gender	4.22	1.43; 12.5	0.009
Not Married	3.38	1.04; 10.9	0.04
Cough in the morning	53.8	10.7; 272	<0.001
Cough during the day	8.66	1.80; 41.8	0.007
Wheeze during the day	35.8	4.82; 267	<0.001
Nagelkereke R^2= 62%; Hosmer & Lemeshow = 0.926; 84.4% of the subjects correctly classified.			

(c)- Third regression- N =201 – COPD patients vs. asthma patients as dependent variable			
SP-D (above the median)	2.53	1.29; 4.96	0.007
Male gender	2.84	1.44; 5.62	0.003
Age	1.04	1.02; 1.07	<0.001
Cough in the morning	8.70	3.29; 23.0	<0.001
Nagelkereke R^2= 39%; Hosmer & Lemeshow = 0.867; 76.1% of the subjects correctly classified.			

Abbreviations: aOR, Adjusted Odds Ratio; CI, Confidence Interval; SP-D, Surfactant Protein-D; COPD, Chronic Obstructive Pulmonary disease.

Table 4: Coordinates of the receiver-operating characteristic curve for all COPD patients and controls

COPD positive if ≥ (a)	Sensitivity	1 – Specificity
5.0000	1.000	1.000
6.5000	0.986	0.913
8.0000	0.986	0.772
9.5000	0.958	0.671
10.5000	0.931	0.470
11.5000	0.889	0.342
12.5000	0.889	0.336
13.5000	0.861	0.235
15.5000	**0.764**	**0.107**
17.5000	0.694	0.087
19.0000	0.694	0.074
20.5000	0.681	0.060
22.5000	0.667	0.047
24.5000	0.639	0.047
26.5000	0.597	0.040
36.5000	0.569	0.040
45.5000	0.556	0.040
47.0000	0.542	0.034
48.5000	0.542	0.027
49.5000	0.486	0.020

51.5000	0.431	0.013
56.5000	0.403	0.013
62.0000	0.306	0.013
67.5000	0.278	0.013
71.5000	0.264	0.013
73.0000	0.250	0.013
74.5000	0.236	0.013
75.5000	0.194	0.000
77.0000	0.181	0.000
78.5000	0.153	0.000
80.0000	0.125	0.000
81.5000	0.111	0.000
83.5000	0.097	0.000
85.5000	0.083	0.000
87.5000	0.069	0.000
101.5000	0.056	0.000
119.5000	0.042	0.000
127.0000	0.028	0.000
130.0000	0.000	0.000

Abbreviation: COPD, Chronic Obstructive Pulmonary Disease.

Table 5: Coordinates of the receiver-operating characteristic curve for ever smokers COPD patients and controls.

COPD positive if ≥ (a)	Sensitivity	1 - Specificity
6.0000	1.000	1.000
8.5000	1.000	0.759
10.5000	0.981	0.724
12.0000	0.963	0.586
13.5000	0.907	0.368
15.0000	0.907	0.356
16.5000	0.889	0.287
18.5000	**0.778**	**0.115**
21.5000	0.685	0.069
24.5000	0.667	0.069
27.5000	0.611	0.057
36.0000	0.574	0.057
44.5000	0.556	0.057
46.5000	0.556	0.034
48.0000	0.537	0.034
49.5000	0.519	0.034
51.0000	0.500	0.034
52.5000	0.481	0.034
54.5000	0.463	0.034

57.5000	0.426	0.034
60.0000	0.407	0.034
62.5000	0.389	0.023
64.5000	0.370	0.023
66.0000	0.315	0.011
69.0000	0.241	0.000
72.5000	0.204	0.000
77.0000	0.093	0.000
95.0000	0.056	0.000
113.0000	0.037	0.000
117.0000	0.000	0.000

Abbreviation: COPD, Chronic Obstructive Pulmonary Disease.