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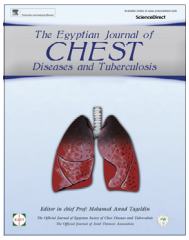

ORIGINAL ARTICLE

Circulating fibrocytes are an indicator of severity and exacerbation in chronic obstructive pulmonary disease

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KEYWORDS

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Abstract *Rationale:* Chronic obstructive pulmonary disease (COPD) is characterized by progressive airflow limitation that is associated with an enhanced inflammatory response in the airways and the lung. The remodeling process in COPD is greatly under the influence of growth factors. Lung fibroblasts in COPD demonstrated alterations in its functional capacity that is mediated by TGF-β1, therefore, could play a role in the pathogenesis of COPD. Fibrocytes are bone marrow derived cells that migrate to the injured sites and differentiate into fibroblast-like cells.

Objectives: To test the hypothesis that assay of circulating fibrocytes may provide a biomarker for exacerbation and severity of COPD.

Methods: Fibrocytes were defined by flow cytometry and quantified in fifty male patients with stable COPD and during exacerbation. We investigated the clinical and prognostic value of fibrocytes by comparison with standard clinical parameters. Thirteen healthy subjects were selected as control.

Results: Fibrocytes were significantly elevated in stable COPD patients ($n = 25$), with a further increase during exacerbation ($n = 25$; $P < 0.001$ vs. control subjects $n = 13$). Correlation analysis between fibrocyte counts and mMRC score, 6-MWT, BODE index, arterial oxygen saturation, pre- and post-bronchodilator FEV_1/FVC, FEV_1, FVC and FEF25–75 showed a direct relationship in COPD patients. There was a direct correlation between fibrocytes with the mMRC score and the serum levels of TGF-β_1 only in COPD patients in exacerbations ($n = 25$).

Conclusions: Fibrocytes are an indicator of COPD exacerbation and might be useful as a clinical (bio) marker for disease progression.

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Introduction

Chronic obstructive pulmonary disease (COPD) is a major cause of morbidity and mortality throughout the world. It is

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a common preventable and treatable disease characterized by persistent airflow limitation that is usually progressive and associated with an enhanced chronic inflammatory response in the airways and the lung to noxious particles or gases. Exacerbations and co-morbidities contribute to the overall severity in individual patients [1]. Worldwide, the most commonly encountered risk factor for COPD is tobacco smoking; current concepts suggest that cigarette smoke leads to an abnormal inflammatory response in the lower respiratory tract that, in turn, leads to tissue damage and destruction [2]. The peri-bronchiolar fibrosis, which develops in the small airways, is believed to be a response to this injury [3]. There are several anatomic lesions that contribute to the reduced airflow found in COPD patients. These include accumulation of mucous secretions, peri-bronchiolar fibrosis, narrowing of small airways and destruction of alveolar walls, which is the defining characteristic of emphysema [4,5]. Destruction of alveolar walls is believed to result from tissue destruction in excess of the capacity of the lung to repair cigarette smoke – induced damage [5,6].

Fibroblasts are believed to be the major cells responsible for the production and maintenance of extracellular matrix (ECM). Alterations in fibroblast functional capacity, therefore, could play a role in the pathogenesis of pulmonary emphysema. Lung fibroblasts from patients with COPD demonstrated less activity in several *in vitro* measures associated with tissue repair. These appeared to be mediated by decreased sensitivity to transforming growth factor-β [6].

Fibrocytes were identified in 1994 as spindle-shaped cells that are likely bone marrow derived. They migrate to sites of tissue injury and can differentiate into fibroblast-like cells [7]. A unique feature of fibrocytes is that they are circulating in the blood stream and are capable of producing ECM components [8]. They express a variety of mesenchymal markers including collagen-1, the leukocyte marker CD45, and the hematopoietic stem cell marker CD34 [7], which is down-regulated with recruitment of the cell to the tissue [9]. The profibrotic cytokine transforming growth factor-β (TGF-β1) stimulates fibrocytes to express α-smooth muscle actin, a typical but nonspecific myofibroblast marker [10], supporting a potential role of these cells in myofibroblast differentiation. Although fibrocytes participate in wound repair, tissue regeneration, and angiogenesis in a positive manner [11,12], they may be negatively involved in the progression of several pulmonary diseases [13,14].

Treatment of COPD is now aimed at immediately relieving and reducing the impact of symptoms, as well as reducing the risk of future adverse health effects such as exacerbations [1]. In COPD, the most common medications prescribed include bronchodilators and glucocorticoids. Despite considerable research effort in defining the effects of glucocorticoid receptor activation in regulating leukocyte function, little is known about its role in modulating fibrocytes. Current evidence suggests that cellular responses to glucocorticoids in fibrocytes may differ from those observed in leukocytes [15].

We hypothesized that in COPD patients with exacerbations, there could be an increased number of circulating fibrocytes compared with stable COPD patients.

Therefore, the objective of our study was to quantify circulating fibrocytes in COPD patients during stable disease and in exacerbations, also to determine a possible biomarker role of fibrocytes in COPD, and finally to examine a potential contribution role of TGF-β_1 and treatment with glucocorticoids on fibrocytes.

Subjects and methods

A prospective study that was conducted at the Chest Department, Kasr El-Aini hospital, Cairo University. Fifty male patients with confirmed diagnosis of COPD (25 stable COPD patients and 25 COPD patients in exacerbation), and 13 healthy male volunteers with no history of smoking as a control group, were involved in the study. Diagnosis of COPD was based on the clinical history, physical examination and spirometric measurements (the presence of a post-bronchodilator $FEV_1/FVC < 0.70$) which confirms the presence of persistent airflow limitation according to the Global Initiative for Chronic Obstructive Lung Disease GOLD 2014 [1]. COPD exacerbation is defined according to GOLD 2014 by an acute worsening of the patient's condition from stable state and beyond normal day-to-day variations, which presents with worsened dyspnea; worsened sputum volume and/or change in its color; or any combination of these symptoms, and requires a change in regular medication [1].

Exclusion criteria were: patients with history of asthma, IPF, cystic fibrosis or active pulmonary tuberculosis. This study was approved by the Human Ethical Committee of Cairo University and all subjects gave informed consent.

The following data were collected:

Clinical data: Age and body mass index (BMI) were obtained for all groups. Smoking status, modified British Medical Research Council scale (mMRC) for dyspnea, presence of co-morbidities, number of previous exacerbations, and steroid therapy in COPD patients, were collected.

6-Minute Walk Test (6-MWT): 6-Minute Walk Distance and measurement of oxygen saturation using pulse oximetry (pre and post 6-MWT), were assessed for all groups. The test was performed according to American Thoracic Society guidelines [16]. For the exacerbation group, it was performed after one week.

Results of spirometry and grading of severity: Measurement of the forced expiratory volume in the first second (FEV_1), forced vital capacity (FVC), FEV_1/FVC, forced expiratory flow 25–75% (FEF25–75%) were obtained for all groups. The results of post-bronchodilator were obtained only for the COPD group. It was performed after one week for patients in exacerbation.

COPD grading according to the severity of airflow limitation (based on Post-Bronchodilator FEV_1) was as follows; GOLD 1(mild) $FEV_1 \geqslant 80\%$ predicted, GOLD 2(moderate) $50\% \leqslant FEV_1 < 80\%$ predicted, GOLD 3(severe) $30\% \leqslant FEV_1 < 50\%$ predicted, GOLD 4(very severe) $FEV_1 < 30\%$ predicted [1].

Calculation of the BODE index: BODE index (body mass index, airflow obstruction, dyspnoea, and exercise capacity) which is a multistage functional scoring system for COPD comprising an assessment of symptoms, a surrogate of the nutritional state, and exercise capacity together with the spirometric measure of airflow (FEV_1) [17]. This multidimensional grading system was shown to be superior over the FEV_1-based GOLD classification [18] for predicting hospitalization and the risk of death among patients with COPD [17,19] (Table 1).

Table 1 Scoring the BODE index.

	0	1	2	3
FEV_1 % pred	⩾65	50–64	36–49	⩽35
6MWD (m)	⩾350	250–349	150–249	⩽149
mMRC	0–1	2	3	4
BMI ($kg.m^{-2}$)	>21	⩽21		

Total BODE index score = 0–10 units.
(FEV_1 % pred = predicted amount as a percentage of the forced expiratory lung volume in the first second; 6MWD = six minute walking distance; mMRC = modified medical research council dyspnea scale; BMI = body mass index) [17].

Results of arterial blood gases (ABG): ABG was done only for COPD patients. Pao_2 < 60 mmHg with or without $Paco_2$ > 50 mmHg when breathing room air indicates severity of an exacerbation.

Separation of fibrocyte from peripheral blood samples: Thirty milliliters of whole blood was obtained on the routine evaluation of stable COPD patients and the control group; and within 24–48 h for the COPD patients with acute exacerbation. Peripheral blood mononuclear cells (PBMCs) were separated from fresh whole blood by standard density-gradient centrifugation, using Ficoll-Paque (Pharmacia Biotechnology, Uppsala, Sweden) at 500 rpm for 20 min at room temperature. After centrifugation, the top plasma layer (i.e. the mononuclear cells at the interface) was harvested and kept frozen in sodium chloride lysis solution for further analysis for TGF-β_1.

Then, the precipitation was diluted 1:1 with sterile phosphate buffered saline (PBS) before to layering over the Ficoll-Paque. The PBMCs at the interface were carefully aspirated with spinning at 1600 rpm for 30 min. The cells were then washed three times with PBS and resuspended in Dulbecco's Modified Eagle Medium (DMEM). The nonadherent mononuclear cell fraction was separated by centrifugation, resuspended in DMEM and mixed with red cell lysis solution to deplete red cells by a second Ficoll-Paque centrifugation. T cells were further depleted with anti-CD34 monoclonal antibody using cell sorting apparatus [20].

Magnetic separation: Freshly isolated peripheral blood cells were stained with anti-CD45 Antibodies coupled to magnetic beads (Miltenyi Biotech, Auburn, CA). Labeled cells were then sorted by binding the cell population to positive selection columns using a SuperMacs apparatus (Miltenyi Biotech) according to the manufacturer's instructions. Cells are then washed extensively. CD45 positive cells are retained on the column and can be removed by flushing the column with buffer once it is removed from the magnetic field. CD45 positive cells are collected in the original flow. Their number was determined by counting the cells that were retained on the column by a hemocytometer. Then, flow cytometry staining on this population confirms that these cells were CD45 positive cells [20].

Flow cytometric analysis: Frozen samples were thawed rapidly in warm water to room temperature and washed in 40 ml of FACS buffer (BD Pharmingen, San Diego, CA), and then centrifuged at 1500 rpm for 5 min. Cells were washed and fixed/permeabilized using the Cytofix/Cytoperm kit from (BD Pharmingen). Fibrocytes were detected by flow cytometry (FACScan; BD Biosciences, Mountain View, CA), using both phycoerythrin (PE)-conjugated anti-CD34 or (PE)-conjugated anti-CD45 (clone RAM34, BD Pharmingen). Thus fibrocyte numbers were expressed as a percentage of total leukocyte counts [21].

Collagen1 mRNA analysis: Cellular mRNA was prepared using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Gene expression for col 1 was determined by Real-time PCR (RT-PCR) amplification which was carried out using 10 μL amplification mixtures containing Power SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA USA), equivalent to 8 ng of reverse-transcribed RNA and 300 nM primers, the sequences of PCR primer pairs used for each gene were as follows:col 1 sense, TGGTGC CAAGGGTCTCACTGGC; col 1 anti-sense, GGACCTTG TACACCACGTTCACC; beta actin sense, GTGGGGCTCC CCAGGCACCA; beta actin anti-sense, GCTCGGCCGTG GTGGTGAAGC. Reactions were run on an ABI PRISM 7900 HT detection system (Applied Biosystems). PCR reactions consisted of 95 °C for 10 min (1 cycle), 94 °C for 15 s, and 60 °C for 1 min (40 cycles). Data were analyzed with the ABI Prism sequence detection system software and quantified using the v1·7 Sequence Detection Software from PE Biosystems (Foster City, CA). Relative expression of studied genes was calculated using the comparative threshold cycle method. All values were normalized to the beta actin gene [22].

Measurement of TGF-β_1: Serum TGF-β_1 was measured by using ELISA (quantikine R&D system USA) according to the manufacturer's instructions [23].

Statistical methods

Data were analyzed using SPSS (Statistical Package for Social Sciences; SPSS Inc., Chicago, IL, USA) version 17 for Microsoft windows. Numerical data were presented as mean ± SD. Categorical data were presented as percentage. Comparisons between 3 groups (control, stable, and exacerbation) of numerical data were conducted using ANOVA (ANALYSIS OF VARIANCE) or non-parametric Kruskal–Wallis test as appropriate, paired comparisons were conducted using Tukey's test. Comparisons between 2 groups (stable and exacerbation) of numerical data were conducted using Student's *t*-test or non-parametric Mann–Whitney *U* test as appropriate. Comparisons between 2 groups (stable and exacerbation) of categorical data were conducted using the Chi-square test. Pearson correlation was used for correlation analysis. Probability (*p*-value) < 0.05 was considered significant and highly significant if *P*-value < 0.001.

Results

Patients' characteristics

In total, 25 patients with stable COPD [15 patients were GOLD 1 and 2 (60%) and 10 patients were GOLD 3 and 4 (40%)], 25 COPD patients in exacerbations and 13 healthy control subjects were included in the study. Patients' characteristics are shown in Table 2.

The mean age of the patients with stable COPD was 56.5 ± 10.2 whereas, the mean age of the COPD patients in exacerbations and normal subjects were 60.2 ± 7.9 and 35.1 ± 8.3, respectively. Also, there was no statistical difference in the mean of the BMI between the 3 groups (P = 0.578).

Table 2 Clinical characteristics of subjects.

	Normal subjects (n = 13)	COPD, stable (n = 25)	COPD, exacerbations (n = 25)	p- value
Age, year	35.1 ± 8.3	56.5 ± 10.2	60.2 ± 7.9	0.045
BMI	26.1 ± 4.7	24.3 ± 4.6	25.1 ± 5.7	0.578
No. of cigarettes	0	22.0 ± 10.0	24.8 ± 10.5	0.3
Duration of smoking	0	25.5 ± 7.4	32.5 ± 6.3	0.001
Smoking index	0	574.4 ± 346.0	794.4 ± 308.4	0.004
mMRC scale	0	2.0 ± 0.9	3.0 ± 0.8	< 0.001**
BODE	0	3.2 ± 2.5	6.4 ± 2.0	< 0.001**
No. of exacerbations	0	1.7 ± 1.1	3.4 ± 2.0	0.001
6-MWT distance	489.2 ± 38.4	372.0 ± 86.2	238.8 ± 82.0	< 0.001*
SO_2 pre-6-MWT	97.7 ± 0.6	95.6 ± 1.6	92.0 ± 4.1	< 0.001*
SO_2 post-6-MWT	97.8 ± 0.8	94.70 ± 2.2	88.9 ± 5.9	< 0.001*
$Paco_{2\ mmHg}$		37.5 ± 4.6	44.7 ± 8.6	0.001
$Pao_{2\ mmHg}$		72.4 ± 8.0	59.1 ± 5.9	< 0.001**
Arterial SO_2%		95.4 ± 1.9	90.3 ± 3.0	< 0.001**
Pre-bronchodilators				
FEV_1/FVC	90.2 ± 7.2	58.5 ± 10.5	53.2 ± 13.0	< 0.001*
FVC, %pred	86.0 ± 10.9	73.5 ± 21.3	45.5 ± 11.9	< 0.001*
FEV_1, %pred	92.7 ± 11.7	56.0 ± 22.1	31.7 ± 11.9	< 0.001*
FEF25–75,%pred	97.5 ± 22.0	30.6 ± 17.2	17.6 ± 11.8	< 0.001*
Post-bronchodilators				
FEV_1/FVC		58.5 ± 10.5	53.2 ± 13	0.118
FVC, %pred		78.4 ± 21.4	49.5 ± 12.2	< 0.001**
FEV_1, %pred		58.5 ± 21.2	33.4 ± 12.5	< 0.001**
FEF25–75,%pred		32.9 ± 17.3	18.0 ± 11.4	0.001

Definition of abbreviations: SO_2 = oxygen saturation; $Paco_2$ = arterial carbon dioxide tension; Pao_2 = arterial oxygen tension, FVC = forced vital capacity; FEV_1 = forced expired volume in the first second; FEF = forced expiratory flow. Data are expressed as mean ± standard deviation (SD).*P*-value < 0.05 is considered significant.

P-value < 0.001 is considered of high statistical significance.

* *P*-value < 0.001, compared with patients of COPD (stable and in exacerbations) and normal subjects.

** *P*-value < 0.001, compared with patients of COPD and those in exacerbations.

Higher mean of smoking index was found among COPD patients with exacerbation than stable COPD (794.4 ± 3, 574.4 ± 3; $P = 0.004$). There were 7/25 (28%) patients with different co-morbidities in the stable COPD cases compared to 12/25 (48%) patients in those with exacerbations and with no significant difference ($P = 0.145$). There was statistical difference between the number of previous exacerbations between the stable COPD patients and those in exacerbations ($P = 0.001$). As regards therapy, there were 19 COPD patients in exacerbations receiving intravenous corticosteroids.

There were a marked statistical difference in the dyspnea mMRC score, BODE index, arterial oxygen tension (Pao_2), arterial oxygen saturation (SO_2) and the post-bronchodilator FVC and FEV_1 between patients with stable COPD and those in exacerbations ($P < 0.001$).

There was a highly statistical significant difference between the three groups of the study as regards the following; the mean distance of the 6MWT, the mean of the oxygen saturation before and after the 6-MWT, and the mean of the pre-bronchodilator spirometry measures (FEV_1/FVC ratio, FVC (%predicted value), FEV_1 (%predicted value), FEF25–75 (%predicted value), ($P < 0.001$).

Fibrocyte quantification

The absolute counts per milliliter of blood fibrocytes was highly significantly increased in the blood of COPD patients in exacerbations ($4.2 \pm 2.0 \times 10^4$ cells/ml, $n = 25$; $P < 0.001$) compared to patients with stable COPD ($1.0 \pm 0.5 \times 10^4$ cells/ml, $n = 25$) and the healthy control subjects ($0.7 \pm 0.27 \times 10^4$ cells/ml, $n = 13$). Similarly, when expressed as percentage of circulating fibrocytes, defined as CD34, CD45 and collagen-1–expressing cells they were also highly significantly increased in the blood of COPD patients in exacerbations (25.2 ± 5.7, 19.7 ± 5.9 and 0.97 ± 0.49, respectively) compared to stable COPD patients (13.6 ± 3.7, 8.9 ± 2.7 and 0.38 ± 0.24, respectively) and the healthy control subjects (9.8 ± 3.5, 8.1 ± 2.0 and 0.14 ± 0.06, respectively); $P < 0.001$ (Table 3). Also, the serum levels of TGF-β_1 were significantly higher in COPD patients with exacerbations (440.6 ± 164.0 pg/ml) than in normal subjects (121.5 ± 18.7 pg/ml) and patients with stable COPD (190.5 ± 21.3 pg/ml); $P < 0.001$ (Table 3).

Moreover, when we compared the percentage of CD34, CD45, collagen-1–expressing cells, the absolute counts of blood fibrocytes and the serum levels of TGF-β_1 (25.24 ± 5.71, 19.73 ± 5.94, 0.97 ± 0.49, 4.21 ± 1.99 and 440.61 ± 163.99, respectively) in the blood of COPD patients in exacerbations to stable COPD patients either GOLD 1 and 2 (mild-moderate) [12.75 ± 3.23, 8.71 ± 2.48, 0.29 ± 0.19, 0.83 ± 0.31 and 185.93 ± 21.78, retrospectively] or GOLD 3 and 4 (severe-very severe) [14.93 ± 4.15, 9.12 ± 3.07, 0.53 ± 0.23, 1.23 ± 0.31 and 197.46 ± 19.62, retrospectively] all were significantly highly elevated; ($P < 0.001$) (Table 4).

Table 3 Show the level of different markers in the COPD (stable and in exacerbations) patients and the control group.

		Normal subjects ($n = 13$) *b*	COPD, stable ($n = 25$) *b*	COPD, exacerbations ($n = 25$) *a*	*p*- value
CD34%	Mean ± SD	9.8 ± 3.5	13.6 ± 3.7	25.2 ± 5.7	< 0.001
	Median	9.3	12.6	26.1	
	(Min–Max)	(6.1–19.3)	(8.1–20.4)	(16.4–34.3)	
CD45%	Mean ± SD	8.1 ± 2.0	8.9 ± 2.7	19.7 ± 5.9	< 0.001
	Median	8.1	9.3	18.5	
	(Min–Max)	(6.1–12.1)	(4.1–12.9)	(12.1–31.4)	
Collagen-1–expressing cells%	Mean ± SD	0.14 ± 0.06	0.38 ± 0.24	0.97 ± 0.49	< 0.001
	Median	0.15	0.24	0.83	
	(Min–Max)	(0.05–0.24)	(0.13–0.91)	(0.24–2.03)	
Fibrocyte number ($\times 10^4$ cells/ml)	Mean ± SD	0.7 ± 0.27	1.0 ± 0.5	4.2 ± 2.0	< 0.001
	Median	0.6	0.9	4.2	
	(Min–Max)	(0.3–1.2)	(0.4–2.1)	(1.2–7.0)	
TGF-β_1 (pg/ml)	Mean ± SD	121.5 ± 18.7	190.5 ± 21.3	440.6 ± 164.0	< 0.001
	Median	118	194	394.5	
	(Min–Max)	(96.2–164.3)	(148.2–219.2)	(194.2–659.1)	

Statistics having different letters are considered of highly statistical difference (p-value < 0.001), i.e. COPD patients in exacerbations compared to patients with stable COPD and normal subjects.

Also, the absolute counts of fibrocytes per milliliter of blood were highly significantly increased in the blood of COPD patients in exacerbations receiving intravenous steroids ($4.4 \pm 1.9 \times 10^4$ cells/ml, $n = 19$; $P < 0.001$) compared to those not receiving intravenous steroids ($1.5 \pm 1.4 \times 10^4$ cells/ml, $n = 31$). However, there was no statistical difference when comparing the absolute counts of blood fibrocytes in COPD patients with different co-morbidities ($3.1 \pm 2.4 \times 10^4$ cells/ml, $n = 19$; $P = 0.49$) to those without co-morbidities ($2.2 \pm 2.0 \times 10^4$ cells/ml, $n = 31$).

Correlation between fibrocyte counts and clinical parameters

Correlation analysis between fibrocyte counts and; number of exacerbations, dyspnea mMRC score, BODE, the 6-MWT, the oxygen saturation before and after the 6-MWT arterial oxygen tension (Pao_2), arterial oxygen saturation (SO_2), pre- and post-bronchodilator FEV_1, FVC and FEF25–75 showed a direct relationship with these parameters in both stable COPD patients and those in exacerbations (50 patients) (Fig. 1).

Also, there was a positive correlation between fibrocyte counts and CD34%, CD45%, % of collagen-1–expressing cells and the serum levels of TGF-β_1 in both stable COPD patients and those in exacerbations (50 patients) (Fig. 1).

To assess whether fibrocyte counts would be different depending on the state of COPD patients, correlation analysis between fibrocyte counts and all the above parameters were calculated separately in both stable COPD patients and those in exacerbations. There were a significant inverse correlation of fibrocyte counts with post-bronchodilator FEV_1 and a

Table 4 Show the level of different markers in stable COPD patients (GOLD 1 and 2 [mild and moderate], GOLD 3 and 4 [severe and very severe]) and COPD patients in exacerbation.

		COPD, GOLD 1&2 ($n = 15$) *b*	COPD, GOLD 3&4 ($n = 10$) *b*	COPD, exacerbations ($n = 25$) *a*	*p*- value
CD34%	Mean ± SD	12.75 ± 3.23	14.93 ± 4.15	25.24 ± 5.71	< 0.001
	Median	12.3	14.9	26.1	
	(Min–Max)	(9.20–19.20)	(8.10–20.40)	(16.40–34.30)	
CD45%	Mean ± SD	8.71 ± 2.48	9.12 ± 3.07	19.73 ± 5.94	< 0.001
	Median	9.1	9.8	18.5	
	(Min–Max)	(4.10–12.20)	(4.20–12.90)	(12.10–31.40)	
Collagen-1–expressing cells%	Mean ± SD	0.29 ± 0.19	0.53 ± 0.23	0.97 ± 0.49	< 0.001
	Median	0.22	0.58	0.83	
	(Min–Max)	(0.13–0.69)	(0.21–0.91)	(0.24–2.03)	
Fibrocyte number ($\times 10^4$ cells/ml)	Mean ± SD	0.83 ± 0.31	1.23 ± 0.31	4.21 ± 1.99	< 0.001
	Median	0.82	1.1	4.2	
	(Min–Max)	(0.41–1.50)	(0.62–2.10)	(1.20–7.1)	
TGF-β_1 (pg/ml)	Mean ± SD	185.93 ± 21.78	197.46 ± 19.62	440.61 ± 163.99	< 0.001
	Median	193.6	204.6	394.5	
	(Min–Max)	(148.20–214.20)	(157.20–219.20)	(194.20–659.10)	

Statistics having different letters are considered of highly statistical difference (p-value < 0.001), i.e. COPD patients in exacerbations compared to stable COPD patients GOLD 1 and 2 and GOLD 3 and 4.

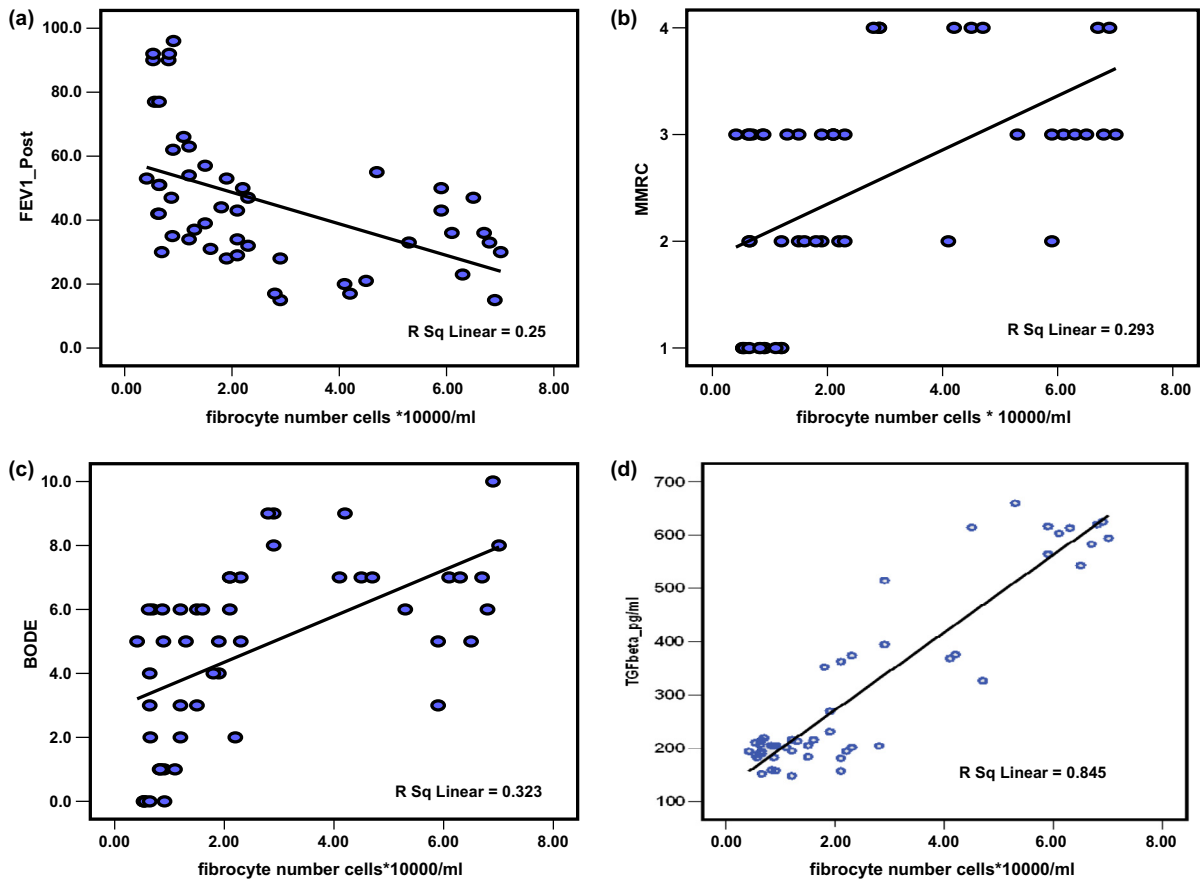

Figure 1 (a) Correlation of fibrocyte counts and Post bronchodilator FEV_1 (b) correlation of fibrocyte counts and mMRC (c) correlation of fibrocyte counts and BODE (d) correlation of fibrocyte counts and TGF-β_1 in COPD patients ($n = 50$). Two tailed Pearson test was used. All correlations were significant at the level 0.01 level (2-tailed).

significant positive correlation with BODE index in stable COPD patients. Moreover in the exacerbation group the same correlations were present; however they did not reach a statistical significance (Fig. 2). Also there was no difference in correlation of fibrocyte counts with post-bronchodilator FVC, and the 6-MWT when comparing stable COPD patients and those in exacerbations. However, there was a direct correlation between fibrocyte counts with the mMRC dyspnea score and the serum levels of TGF-β_1 only in COPD patients in exacerbations (Fig. 3).

Finally, there was also no relationship between fibrocytes and age, BMI or smoking index in all COPD patients (50 patients). However, there was a correlation between age and the number of exacerbations in both stable COPD patients and those in exacerbations.

Discussion

The main finding of our study was the presence of increased percentage of CD34, CD45, Collagen-I expressing cells, numbers of circulating fibrocytes and elevated serum levels of TGF-β_1 in the peripheral blood of COPD patients in exacerbations compared to stable COPD patients and normal subjects with direct correlation between fibrocyte counts and the serum levels of TGF-β_1 only in COPD patients in exacerbations.

In a study done by Togo et al. [6], they cultured fibroblasts from lung tissue obtained from individuals undergoing thoracotomy and were characterized *in vitro*. They found that fibroblasts from patients with COPD produced more TGF-β_1 compared with similarly aged control subjects with similar smoking histories. They concluded that fibroblasts from patients with COPD manifested reduced response to TGF-β_1 and have diminished repair responses that could contribute to the development of emphysema in these subjects. They also added that the mechanisms that account for the differences in fibroblast function in the subjects with COPD compared with the control subjects are not yet defined and that cigarette smoke exposure is unlikely to account for the differences, because the two groups had similar smoking histories; despite the evidence that acute exposure of fibroblasts to cigarette smoke has been reported to inhibit fibroblast repair functions [24,25]. In our study, we also found that there was no correlation between fibrocytes and the smoking index in COPD patients.

Fibrocytes have been found in patients with various fibrotic disorders and are involved in the pathogenesis of bronchial asthma [21] and lung fibrosis [26]. Wang et al. [27] found increased numbers of fibrocytes and increased levels of TGF-β_1 in the serum of patients with chronic obstructive asthma compared with asthmatic patients with normal lung function and normal subjects. They found a significant

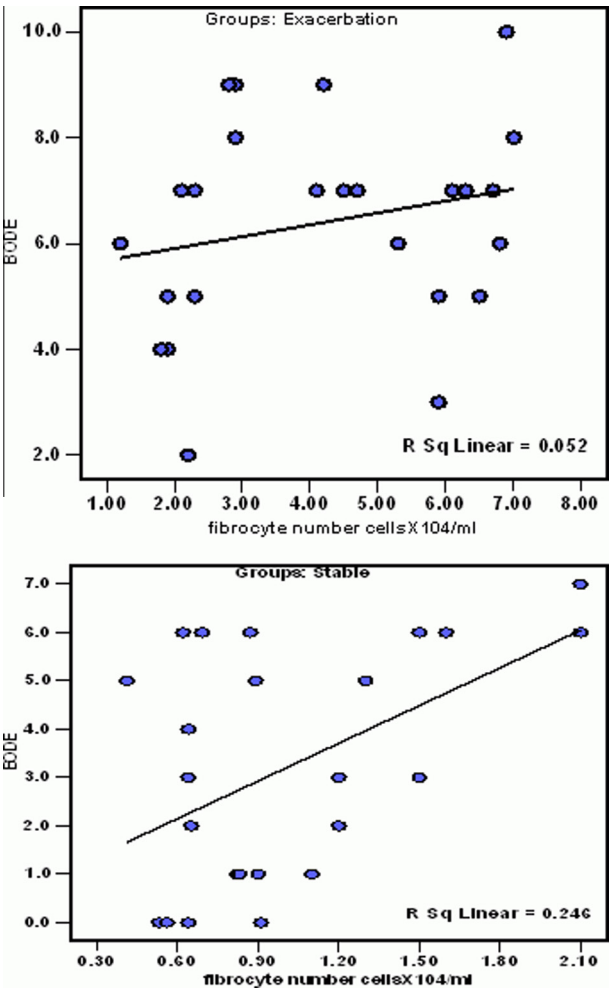

Figure 2 (a) Correlation of fibrocyte counts and BODE in COPD patients in exacerbations ($n = 25$). (b) Correlation of fibrocyte counts and BODE in stable COPD patients ($n = 25$). Two tailed Pearson test was used. Correlations were significant at the level 0.01 level (2-tailed). There was no difference in correlation between stable COPD patients and those in exacerbations.

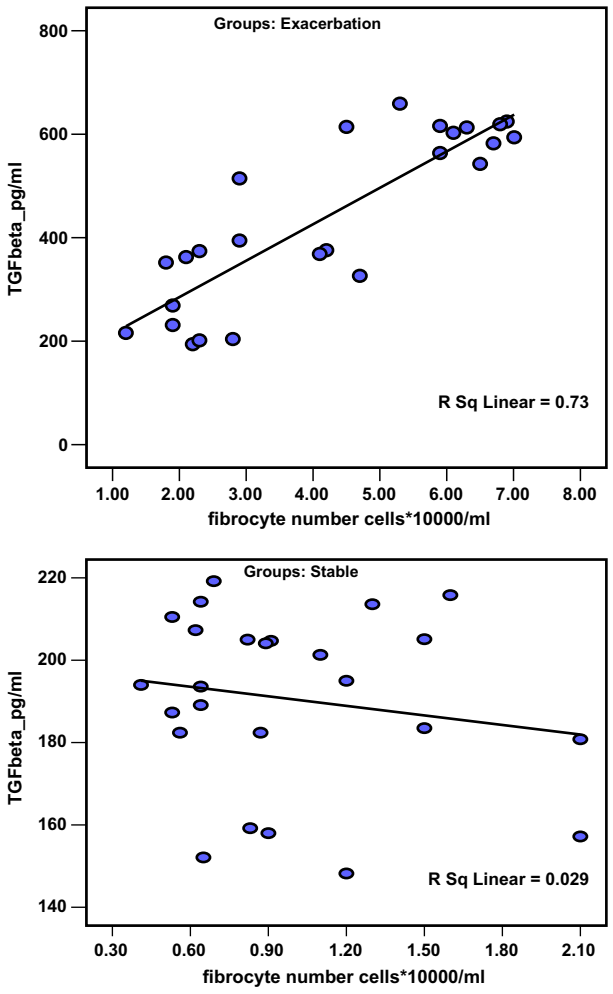

Figure 3 (a) Correlation of fibrocyte counts and TGF-β_1 in COPD patients in exacerbations ($n = 25$). (b) Correlation of fibrocyte counts and TGF-β_1 in stable COPD patients ($n = 25$). Two tailed Pearson test was used. Positive correlation was significant at the level 0.01 level in COPD patients in exacerbations ($n = 25$) (2-tailed).

correlation between the percentage of circulating fibroblasts and serum TGF-β_1 in their patients with chronic obstructive asthma and the yearly decline in FEV_1. This finding is not surprising because TGF-β_1 plays an important role in the differentiation of fibrocyte-like progenitor cells and enhances the differentiation of CD45, CD34 and Collagen-I expressing cells into fibroblasts/myofibroblasts. In these cells, TGF-β_1 also stimulates expression of a number of proinflammatory and fibrogenic cytokines, such as tumor necrosis factor (TNF-a), platelet-derived growth factor (PDGF), IL-1b, or IL-13, thereby further enhancing collagen production and thickening of the sub-basement membrane observed in patients with asthma. The mechanism by which remodeling may link to the progressive decline in lung function leading to chronic airflow obstruction is seen in some patients with asthma [28,29].

Regarding that the clinical management and staging of COPD which relies on monitoring of pulmonary function tests (PFT) and exercise capacity, usually by 6MWT we correlated the number of fibrocytes to those parameters. we found that there was a direct correlation between the number of fibrocytes and the pre- and post-bronchodilator FEV_1/FVC, FEV_1, FVC, FEF25–75, 6-MWT, the oxygen saturation before and after the 6-MWT in all COPD patients but when correlating those parameters to the state of COPD there was no difference between stable COPD and patients in exacerbations.

Recently, the incorporation of the subjective variable 'dyspnea' and the individual exercise capacity, the BODE index was used more as it is thought that it is closely related to the individual subjective consequences of COPD than lung function alone [17–19]. In our study there was a positive correlation between the number of fibrocytes and the mMRC score and BODE in all COPD patients. But when correlating those parameters to the state of COPD only the mMRC score showed a direct relationship to COPD patients in exacerbations.

Also, Moeller et al. [30] studied the effect of fibrocytes as an indicator of poor prognosis in IPF patients. They demonstrated a threefold increase in circulating fibrocytes ($CD45^{+-}$

Col-1^{+} cells) in patients with stable IPF compared with healthy control individuals. They also found that during episodes of acute disease exacerbation, fibrocyte counts further increased to an average of 15% of peripheral blood leukocytes and fell back to pre-exacerbation levels in the patients who recovered. They also compared their findings to a study on a small group of patients with IIP, who had increased fibrocytes in their circulation during stable disease [31].

When Nihlberg et al. [29] studied the effect of steroids on fibrocytes in asthmatic patients, they found that in steroid-naive patients with mild asthma, an increased number of fibrocytes was observed in the bronchial mucosa as well as in the bronchoalveolar lavage fluid; and the number of submucosal fibrocytes had been correlated with the thickness of the subbasement membrane. However, in our study, fibrocytes were significantly increased in the blood of COPD patients receiving intravenous steroids, a finding that could be explained that those patients were in exacerbations with already elevated counts of blood fibrocytes.

To date, FEV_1 changes over time may be the best functional marker in COPD, even if the change may not necessarily reflect progression of the underlying disease. Our study was able to demonstrate a potential relationship between mMRC score, FEV_1 changes, 6MWT, BODE, oxygen saturation and the change in fibrocyte percentage in COPD patients. Even stable COPD patients GOLD 3 and 4 showed a higher fibrocyte counts and in these patients; though lacking clinical signs of deterioration, they might be at greater risk of exacerbation or rapid progression.

Also, we speculate that the increase in fibrocyte counts may precede the clinical and functional decline, but because of the relatively low subject numbers, larger and long-term studies are needed to examine this hypothesis.

Despite numerous *in vitro* and *in vivo* studies, the biology of mesenchymal progenitor cells and circulating fibrocytes is complex and incompletely understood. On the one hand, they seem to be able to produce cytokines, growth factors and extra cellular matrix ECM proteins [32,33]. On the other hand, there is evidence that these cells are involved in abnormal scar formation in models of wound repair, possibly through fibrocyte-mediated T-cell activation and could play a role in the pathogenesis of pulmonary emphysema [34–36]. However, the present study has confirmed that fibrocytes are present in the blood of patients with COPD and even more so during acute exacerbation of the disease, which allows the examination of their potential as biomarkers, regardless of understanding their biological properties in detail.

In conclusion, we found increased counts of fibrocytes in the blood of COPD patients with exacerbations compared with stable COPD patients and healthy control subjects. Fibrocytes did not correlate with clinical parameters such as age, BMI and smoking index but were predictive of severity as regards mMRC score, FEV_1 changes, BODE, 6MWT and oxygen saturation. This study suggests a potential role for assaying fibrocytes as an independent prognostic biomarker for COPD; however, larger studies are needed to confirm or refute these findings.

Conflict of interest

We have no conflict of interest to declare.

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