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Association between cytokines in induced sputum and severity of chronic obstructive pulmonary disease

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Summary Cytokines are known to be increased in induced sputum in chronic obstructive pulmonary disease (COPD). In this study, the relationship between the levels of interleukin-6 (IL-6), interleukin-8 (IL-8), and tumour necrosis factor-α (TNF-α) in induced sputum of patients with exacerbation of COPD, and the severity of the disease, pulmonary function tests (PFT), arterial blood gases (ABG) were studied.

Twenty-four patients with exacerbation of COPD were included in the study. The patients were grouped according to their PFT into two as: Group 1 (FEV_1 below 50% of the predicted value, severe–very severe COPD, $n = 12$) and, Group 2 (FEV_1 above 50% of the predicted value, mild–moderate COPD, $n = 12$). The levels of IL-6, IL-8 and TNF-α in induced sputum of the subjects were measured.

The mean levels of IL-6, IL-8 and TNF-α in induced sputum were found to be higher in Group 1 (severe–very severe COPD) than in Group 2 (mild–moderate COPD). The differences in IL-6 and IL-8 levels between groups were statistically significant ($P < 0.05$). A significant correlation was observed between the IL-6 value and FEV_1 ($r = -0.435$, $P = 0.034$), FEV_1/FVC ($r = -0.446$, $P = 0.029$), PaO_2 ($r = -0.711$, $P = 0.000$), SaO_2 ($r = -0.444$, $P = 0.030$) and disease duration ($r = 0.427$, $P = 0.037$), respectively. Also, the level of IL-8 in induced sputum was inversely correlated with FEV_1 ($r = -0.562$, $P = 0.004$), PaO_2 ($r = -0.540$, $P = 0.006$) and SaO_2 ($r = -0.435$, $P = 0.034$). However, all three cytokines were positively correlated with the smoking load ($r = 0.653$, $P = 0.001$; $r = 0.439$, $P = 0.032$; $r = 0.649$, $P = 0.001$).

We conclude, therefore, that in exacerbated COPD cases with greater degrees of obstruction of the airways have higher levels of cytokines in induced sputum. This

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can be interpreted to mean that these cytokines are related to the clinical parameters like the ABG and PFT and seem to be the determinant of the severity of the disease.

Introduction

Chronic obstructive pulmonary disease (COPD) is an important lung disease characterized by progressive airway obstruction due to chronic inflammation.[1] Exacerbations in COPD lead to increases in the number of patients reporting to the hospital and the frequency of admissions. It also affects adversely the quality of life of the patients and restricts their daily activities.[2] COPD exacerbations are responsible for the increase in morbidity and mortality rates attributable to the disease.[3]

Cytokines are extracelluler signal proteins less than 80 kDa formed by various cell types in the body. Interleukin-6 (IL-6) is secreted by monocytes, macrophages, T cells, B cells, fibroblasts, epithelial cells of the airways and endothelial cells. Under the activation of IL-1β and TGF-β, IL-6 is secreted from the smooth muscles of the airways. IL-6 are pleotrophic cytokines with a role in the activation, proliferation and differentiation of T cells. IL-6 also serves as a terminal differentiating factor for B cells, and induces immunoglobins like IgG, IgA and IgM.[4] Interleukin-8 (IL-8), also known as CXCL8, is a CXC chemokine that is a potent chemoattractant for neutrophils. In general, monocytes, tissue and alveolar macrophages, pulmonary epithelium, cells of the smooth muscles of the airways, eosinophils, fibroblasts, and endothelial cells are its important sources.[5] Tumour necrosis factor-α (TNF-α) is produced from several cells including T lymphocytes, mast cells and cells of the airway epithelium. TNF-α controls cellular migration and permeability and also stimulates the secretion of GM-CSF, IL-6 and IL-8. It also activates the transcription factor-κB (NF-κB) which in turn activates the IL-8 gene in epithelial cells and macrophages.[6]

The role inflammation and the cytokines play in the pathogenesis of COPD have attracted more interest in recent times.[4,6,7] In COPD patients elevation of cytokine levels are known to be even more apparent during exacerbations. This situation has been demonstrated by the presence of inflammatory cells and elevated levels of the cytokines in the serum, induced sputum, bronchoalveolar lavage (BAL) fluid and bronchoscopic biopsy specimens.[8–11] Aaron et al.[12] studied the levels of IL-8 and TNF-α in induced sputum among COPD patients during their stable periods, at exacerbation and during the stable period a month after the exacerbation episode. The levels of IL-8 and TNF-α in induced sputum were observed to be higher in the exacerbation period than in the stable period, falling again during the stable period a month afterwards.

Many cytokines are increased or activated in COPD, but their contribution to disease severity is still unknown. In this study, the relationship between the levels of IL-6, IL-8 and TNF-α in induced sputum and the severity of the disease, pulmonary function tests (PFT), arterial blood gases (ABG) were investigated.

Materials and method

A total of 30 male COPD patients reporting to the pulmonary diseases clinic of the Turgut Ozal Medical Center, Inonu University, (Malatya, Turkey) with exacerbation of disease were enrolled. Diagnosis of COPD was made based on the American Thoracic Society and European Respiratory Society criteria,[13] with exacerbation identified according to the definition by Anthonisen et al. which is based on an increase in symptoms of dyspnoea, sputum volume and sputum purulence with or without symptoms of upper respiratory infection and then subdivided depending on the number of symptoms. All patients with COPD exacerbation in our study have all major exacerbation symptoms (type I exacerbation).[14]

The patients had no other pulmonary disease including asthma, bronchiectasia, pneumonia, tuberculosis or lung cancer. All subjects were hospitalized because of type I exacerbation of COPD and they were no medications except for COPD. Each patient was given a brief explanation about the study and their consent for voluntary participation obtained. The clinical history of each patient was obtained after which a through physical examination was conducted. Routine laboratory analysis, echocardiography and radiologic studies were ordered. PFT were performed in all cases by using the Vmax 22 device (SensorMedics Ltd., Yorba Linda, CA, USA) and ABG conducted for each patient breathing room air. A total of six patients were excluded from the study for various reasons: one

patient due to elevated liver enzymes, two patients due to development of bronchospasm during the induction stage for sputum, and for withdrawal of consent by another three patients. Therefore 24 patients therefore completed the study.

The subjects were classified into two groups based on their FEV_1 results. Those with an FEV_1 below 50% of the predicted value were placed in Group 1 (severe–very severe COPD, $n = 12$), while those with FEV_1 above 50% of the predicted value formed Group 2 (mild–moderate COPD, $n = 12$).

Before the commencement of the treatment for the exacerbation induced sputum samples were taken from patients by ultrasonic nebulizators (Hirtz, Hico-Ultrasonat 806 EH, Köln-Germany). For this purpose, patients were made to rinse their mouths with water after inhalation of a 3% saline solution for 5–7 min and then asked to expectorate deeply for the 2 ml induced sputum samples to be collected. In patients in whom adequate amount of sample could not be collected saline inhalation was repeated at the same dose and subsequently increased (at a concentration of 4.5%).[15] The samples that were collected into a wide-covered sterile 50 cc polypropylene plastic cups were immediately transferred to the laboratory after labelling for processing. The induced sputum samples were analysed within an hour. The sputum samples were prevented from contamination by saliva by filtering through a double-layered sterile gauze immediately after collection. With the aid of a forceps and spatula approximately 1–1.5 g was taken from each sample and transferred into 10 ml sterile polypropylene tubes with predetermined weights and reweighed. After the addition of a phosphate buffer solution (PBS) that weighed nine times their fresh weight 5–6 pieces of silicone glass beads were introduced into each tube and stirred with a vortex for 10 min until a homogenized solution was obtained. The solution was thereafter subjected to centrifugation at +4 °C for 10 min at 1000*g*. The resulting supernatant was transported into polypropylene tubes in aliquots of 1 ml. After the addition of 10 μl 100 mM phenyl methyl sulfonyl fluoride (PMSF) into each tube they were covered and turned upside down several times to ensure adequate mixing. Until analysed they were stored at −70 °C.[16]

Table 1 Basic properties and laboratory values of patients in Groups 1 and 2.

	Group 1 ($FEV_1 < 50\%$)	Group 2 ($FEV_1 > 50\%$)	*P* value
Subjects, *n*	12	12	
Age (years)	59.3 ± 11.7	59.8 ± 9.3	NS
Duration of disease (years)	13.8 ± 7.7	13.3 ± 7.0	NS
Smoking load (pack-years)	41.4 ± 21.1	28.6 ± 14.0	NS
Smoking status (yes/ex/never)	7/5/0	8/4/0	NS
IL-6 (pg/ml)	697.8 ± 662.8	216.1 ± 350.8	0.037
IL-8 (pg/ml)	10054.3 ± 2330.2	7159.3 ± 2973.2	0.014
TNF-α (pg/ml)	134.6 ± 140.7	96.3 ± 158.1	NS
FVC (% predicted)	63.5 ± 18.8	90.9 ± 20.9	0.003
FEV_1 (% predicted)	33.8 ± 9.7	61.9 ± 18.8	0.000
FEV_1/FVC (%)	43.7 ± 9.1	50.0 ± 9.7	NS
$FEF_{25\%-75\%}$	13.3 ± 4.9	27.8 ± 15.1	0.005
PH	7.39 ± 0.8	7.45 ± 0.4	0.04
PaO_2 (mmHg)	45.8 ± 7.1	52.6 ± 6.2	0.021
$PaCO_2$ (mmHg)	44.0 ± 7.4	40.8 ± 8.5	NS
SaO_2 (%)	79.6 ± 9.2	85.7 ± 6.2	0.002
HCO_3^- (mmol/l)	29.0 ± 7.0	30.1 ± 5.4	NS
Hemoglobin (g/dl)	15.1 ± 2.4	15.4 ± 1.3	NS
Hematocrit (%)	47.1 ± 8.2	47.9 ± 5.6	NS
BUN (mg/dl)	22.8 ± 13.6	18.4 ± 10.4	NS
Leukocyte count (10^9/l)	12366.7 ± 5261.4	9375.0 ± 3252.7	NS
Creatinine (mg/dl)	1.0 ± 0.3	0.8 ± 0.2	NS
Albumin (mg/dl)	2.6 ± 0.2	3.2 ± 0.5	NS
PAP_s (mmHg)	38.6 ± 25.9	30.6 ± 19.6	NS

Data presented as mean ± SD (standard deviation) or *n*, unless otherwise stated. IL-6: interleukin-6, IL-8: interleukin-8, TNF-α: tumor necrosis factor-α, FVC: forced vital capacity, FEV_1: forced expiratory volume in 1 s, $FEF_{25\%-75\%}$: forced mid-expiratory flow, pH: arterial blood pH, PaO_2: arterial oxygen tension, $PaCO_2$: arterial carbon dioxide tension, SaO_2: arterial oxygen saturation, HCO_3^-: arterial bicarbonate, BUN: blood urea nitrojen, PAP_s: systolic pulmonary artery pressure), NS: not significant.

The IL-6, IL-8, TNF-α levels were measured by the chemiluminiscence method using an Immulite-1000 analyzer and special kits (DPC [Diagnostic Product Co., CA, USA]). The lower limit of detection for these assays was 5 pg/ml for IL-6, 2 pg/ml for IL-8 and 5 pg/ml for TNF-α. The inter- and intra-assay coefficient of variation of these all assays was less than 10%. The samples which were higher than detection limits of cytokines were reanalysed with a dilution of 0.9% serum saline solution for one times weight.

The patients were compared for demographic and laboratory parameters of subjects. The data obtained were analysed with the SPSS version 10.0 (Chicago, IL, USA) statistical program. Data are expressed as means and standart deviation. Differences between groups were analysed using Student's *t*-test. Correlations between different parameters were evaluated using the Pearson's correlation test. The significance level was considered as $P<0.05$.

Results

The demographic properties and laboratory values of the subjects in Groups 1 and 2 are shown in Table 1. The differences of values of FVC, FEV_1, $FEF_{25\%-75\%}$, pH, PaO_2 and SaO_2 between groups revealed statistically significant ($P<0.05$). The average induced sputum IL-6 level for Groups 1 and 2 was 697.8 ± 662.8 and 216.1 ± 350.8 pg/ml, respectively ($P=0.037$). The level of IL-8 in the induced sputum was 10054.3 ± 2330.2 pg/ml for Group 1 and 7159.3 ± 2973.2 pg/ml for Group 2 ($P=0.014$). The levels of TNF-α in induced sputum were found to be 134.6 ± 140.7 and 96.3 ± 158.1 pg/ml for Groups 1 and 2, respectively. No statistically significant difference was observed between the groups in the TNF-α levels ($P>0.05$).

As shown in Fig. 1a and b, the levels of IL-6 and IL-8 in induced sputum correlated negatively with FEV_1 ($r=-0.435$, $P=0.034$; $r=-0.562$, $P=0.004$). In addition, the levels of IL-6 in induced sputum significantly correlated, respectively, with FEV_1/FVC ($r=-0.446$, $P=0.029$, Fig. 1c) and disease duration ($r=0.427$, $P=0.037$, Fig. 1d). Also, the levels of IL-6 and IL-8 in induced sputum correlated negatively with PaO_2 ($r=-0.711$, $P=0.000$, Fig. 1e; $r=-0.540$, $P=0.006$, Fig. 1f) and SaO_2 ($r=-0.444$, $P=0.030$, Fig. 1g; $r=-0.435$, $P=0.034$, Fig. 1(h). However, there was a significant correlation between levels of all three cytokines and smoking load (pack-years) ($r=0.653$, $P=0.001$; $r=0.439$, $P=0.032$; $r=0.649$, $P=0.001$, Fig. 2).

Discussion

In COPD there is a chronic inflammatory process within the airways that increases during an acute episode.[1,6] The induced sputum method, a non-invasive method adequate enough in demonstrating inflammation in the airways such as that occuring in COPD has been shown in earlier studies to be a reliable method.[15–18] In our study, the cytokine levels in patients with exacerbation of COPD have been found to relate to the PFT, ABG, as well as to the severity of disease.

Keatings et al.[19] found high IL-8 levels in induced sputum of COPD patients. In another study, Yamamoto et al.[20] found higher IL-8 levels in the sputum of COPD patients than that in asthmatic patients and normal controls. In their study, Vernooy et al.[21] found higher induced sputum IL-8 levels in 18 COPD patients compared to their 17 smoking control subjects. Keatings et al.[19] and Yamamoto et al.[20] demonstrated a positive correlation between the IL-8 level and the neutrophil proportions. This could not be verified in our study because characterization of cellular components of the sputum was not performed. On the other hand, all patients in this study had all the major symptoms of exacerbations as an increase in symptoms of dyspnoea, sputum volume and sputum purulence (type I exacerbation).[14,22] Gompertz et al.[23] suggest that exacerbations of COPD defined by sputum colour, differ in the degree of bronchial and systemic inflammation. They found purulent exacerbations were associated with marked bronchial and systemic inflammation and bacterial infection. Another lacks of present study, the bacteriology or viral cultures of sputum was not processed, so this papers is not given any information about levels of airway inflammation and bacterial infections or colonizations. We found the cytokines levels in the induced sputum more higher in severe or very severe COPD patients than mild or moderate COPD patients. It has been suggested that these cytokines play a role in airway inflammation and severity of disease.

With each episode of exacerbation there is gradual deterioration in the respiratory functions of the patient. In those patients with frequent exacerbations the fall in FEV_1 is much greater than it is in those with less frequent exacerbations.[24] Frequent exacerbations increase the severity of the respiratory tract inflammation. Bhowmik et al.[8] found that the levels of IL-6 and IL-8 in induced sputum of patients with three or more episodes per year were higher than those with less frequent attacks. In contrast, Gompertz et al.[25] found no significant differences between those with three or

more episodes of exacerbation per year than those with less attacks. Soler et al.[26] demonstrated a negative correlation between the FEV_1 and the IL-6 and IL-8 levels in BAL fluid. In contrast to these studies, however, Bhowmik et al.[8] failed to establish any relationship between the induced sputum IL-6 and IL-8 levels and the PFT parameters and the time for improvement in symptoms and PEF values in the exacerbation period. Yamamoto et al.[20] reported a negative correlation between the IL-8 levels in induced sputum and the FEV_1/FVC ratio in COPD. Similarly we found a negative correlation between the levels of IL-6 in induced sputum and FEV_1/FVC ratios in this study. Also, FEV_1 of the predicted value was inversely correlated with the levels of IL-6 and IL-8 in induced sputum, but not

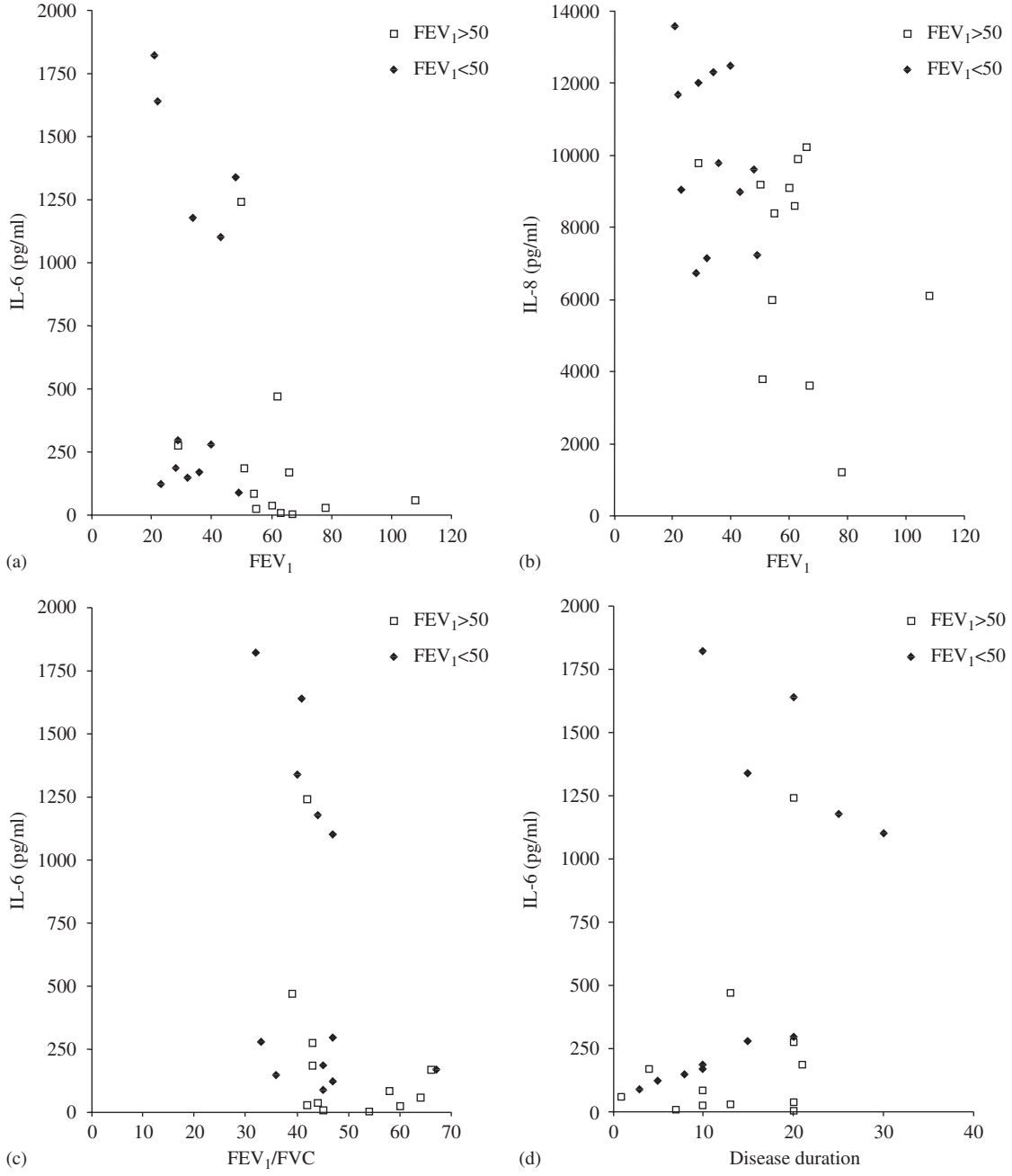

Figure 1 Correlations between (a) the levels of IL-6 and FEV_1 ($r = -0.435$, $P = 0.034$); (b) the levels of IL-8 and FEV_1 ($r = -0.562$, $P = 0.004$); (c) the levels of IL-6 and FEV_1/FVC ($r = -0.446$, $P = 0.029$); (d) the levels of IL-6 and disease duration ($r = 0.427$, $P = 0.037$); (e) the levels of IL-6 and PaO_2 ($r = -0.711$, $P = 0.000$); (f) the levels of IL-8 and PaO_2 ($r = -0.540$, $P = 0.006$); (g) the levels of IL-6 and SaO_2 ($r = -0.444$, $P = 0.030$); (h) the levels of IL-8 and SaO_2 ($r = -0.435$, $P = 0.034$).

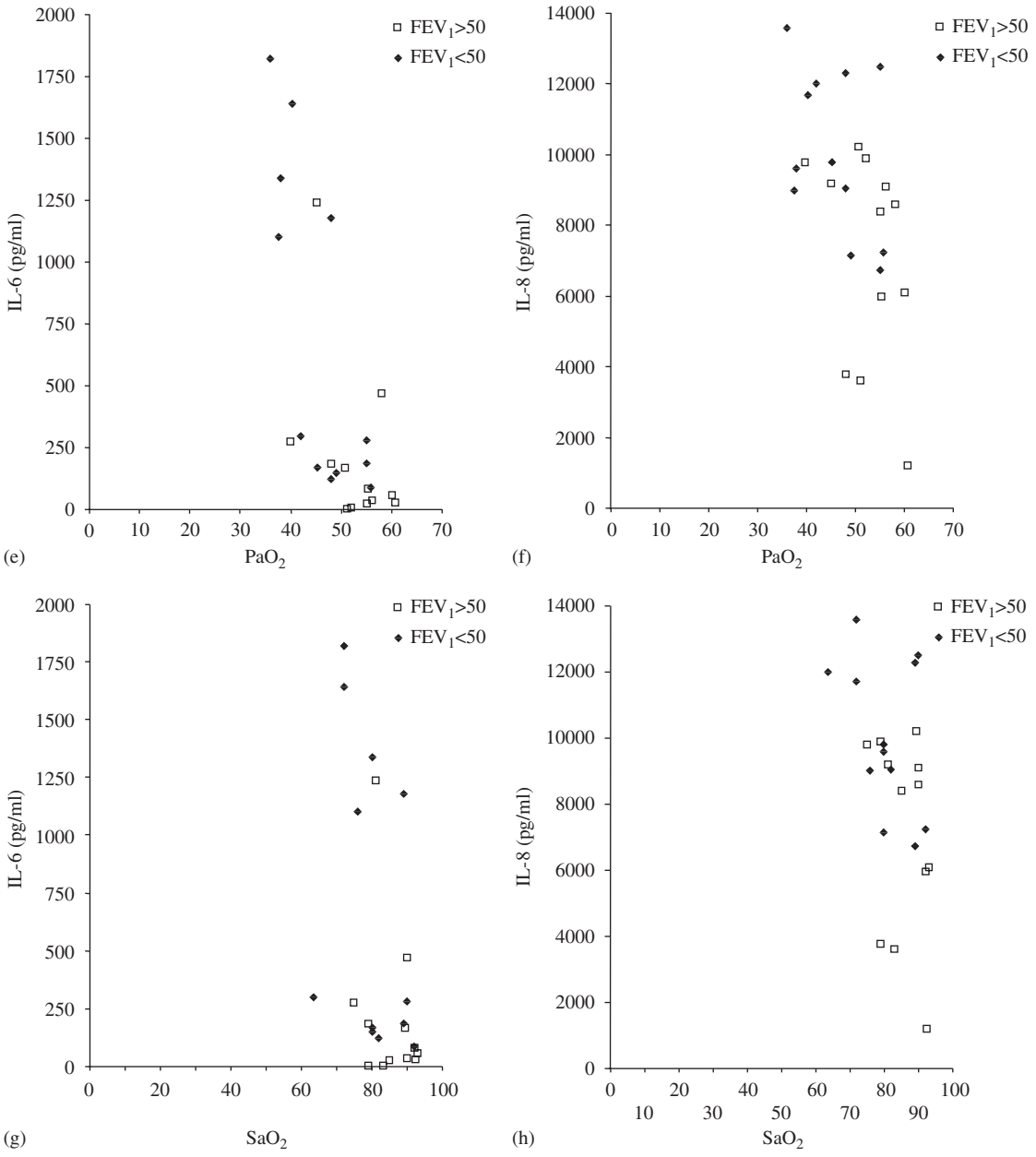

Figure 1 (*Continued*)

TNF-α levels. This indicates that the levels of IL-6 and IL-8 in induced sputum might be related to the severity of the respiratory tract obstruction. And we think that presence of high levels of cytokines in induced sputum increases inflammation in the airways that in turn increases the susceptibility to exacerbations.

With hypoxia IL-6 and IL-8 are secreted from the endothelial cells.[27,28] In addition, hypoxia is thought to be related to activation of the TNF-α system in COPD patients.[29] Yasuda et al.[30] found that although the difference was not statistically significant, the TNF-α and IL-6 levels among severe COPD patients with hypoxemia was higher than their non-hypoxic controls who had mild–moderate COPD. We found severe and very severe patients who are more hypoxemic patients had higher cytokines levels. However, there was a relation between the levels of IL-6 in induced sputum and hypoxemia, might due to increased inflammation associated with hypoxemia.

Cigarette smoking is the most important cause of COPD. However, some smokers develop COPD and the reason is still unknown.[1] Bhowmik et al.[8] established a correlation between the levels of IL-6 and IL-8 in induced sputum and the pack-years of

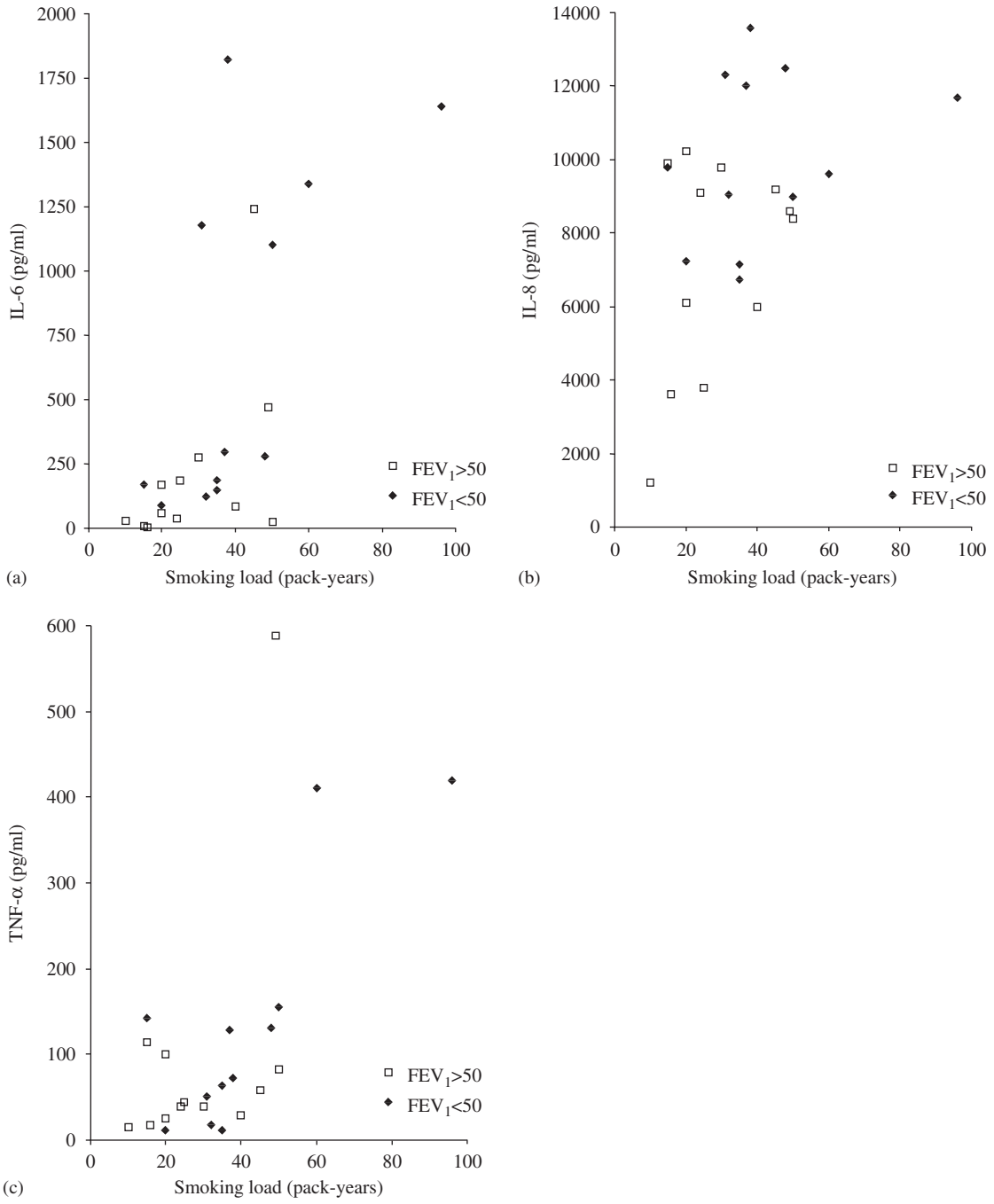

Figure 2 Correlations between (a) the levels of IL-6 and smoking load (pack-years) ($r = 0.653$, $P = 0.001$); (b) between the levels of IL-8 and smoking load (pack-years) ($r = 0.439$, $P = 0.032$); (c) between the levels of TNF-α and smoking load (pack-years) ($r = 0.649$, $P = 0.001$).

cigarettes smoked. Wang et al.[31] measured the levels of IL-6 and IL-8 in induced sputum in 20 stable COPD patients and compared them with smoking and non-smoking control groups. The IL-8 and TNF-α levels in the COPD group were found to be significantly higher than the smoking and non-smoking groups with the IL-8 level in the smoking group also significantly higher than the non-smoking group. The IL-6 levels, however, did not show any significant differences between the groups. In our study a positive correlation was established between the levels of all three cytokines and the smoking load (pack-years). This indicates that higher smoking load had higher cytokine levels in their induced sputum. The initiation of cytokines release in COPD is likely to be due to the direct

effect of cigarette smoking. Also, our study highlighted a close link between smoking and levels of cytokines in induced sputum and therefore smoking needs to be prevented. On the other hand, correlations examined in induced sputum suggest that those patients with a longer history of disease duration had higher cytokines levels. For the determination of disease duration, we used the date of first visit to a doctor for chronic complaints of cough, dyspnea and sputum production.

We therefore conclude that, in our study, COPD patients with more marked obstruction had higher IL-6, IL-8 and TNF-α levels in their induced sputum samples. In addition to its determinant role in the pathogenesis of COPD the levels of IL-6 and IL-8 in induced sputum were found to be directly related to the ABG and PFT, parameters that determine the severity of disease. The airway inflammation in those patients with higher cytokine levels increased leading to an increase in the rate of exacerbations. Reducing the rate of exacerbation episodes in COPD will no doubt result in a marked fall in the morbidity and mortality of the disease. Current treatment is centered on the use of bronchodilators. As the importance of inflammation in the pathogenesis of COPD emerges treatment of COPD, just as in asthma will definitely be geared toward the control of inflammation. By contrast, the inflammatory process in COPD appears to be resistant to corticosteroids, indicating that new anti-inflammatory treatments will be needed. It is our opinion, therefore, that anti-cytokine agents to be developed will be an important leap forward in the prevention of COPD and its progression as well as minimalizing severity of disease.

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