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Cytokine levels in the sera of patients with idiopathic pulmonary fibrosis

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Summary
Background: Idiopathic pulmonary fibrosis (IPF) is a fibroproliferative disorder. Cytokines contribute an important but yet undefined role to its pathogenesis.
Objectives: The present study aims to compare serum levels of cytokines involved in Th-1 and Th-2 immunity, such as interleukins (IL) IL-2, IL-4, IL-8, IL-10, interferon-γ (IFN-γ) and IL-12 (p40) in patients with IPF and healthy volunteers. Twenty patients with IPF and 40 healthy controls (HC) participated.
Methods: Cytokines were assessed by enzyme-linked immunoabsorbent assay (ELISA).
Results: Median values of serum IL-2, IL-8, IL-10, IL-12 (p40) were higher in the IPF than the control group: IPF group: 1.05 U/ml, 12.55, 10.13, 44.17 pg/ml; control group: 0.05 U/ml, 6.91, 0.75, 4.51 pg/ml, respectively ($P < 0.05$). IFN-γ serum levels were lower in the IPF (0.19 pg/ml) than in the control group (0.49 pg/ml). IL-4 values did not differ in a statistically significant way among the groups: 8.40 pg/ml in the IPF group, and 7.46 pg/ml in the control group ($P > 0.05$). IL-4 positively correlated to fast expiratory volume in 1 s (FEV$_1$%) and forced vital capacity (FVC%), while IL-8 negatively correlated to the respective values ($P < 0.005$).
Conclusions: IL-2, IL-8, IL-10 and IL-12 (p40) were found to be elevated in the sera of patients with IPF. IFN-γ was found to be decreased in the sera of patients with IPF.
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Introduction

Idiopathic pulmonary fibrosis (IPF) is a chronic fibroproliferative disorder, which consists of the progressive fibrosis of the interstitial spaces of the lung with subsequent loss of the normal parenchymal architecture.[1] Cytokines are important in the pathogenesis of IPF.[2] Cytokines participate in the Th-1 or Th-2 types of immune response, which are different pathways of immunological reactions, orchestrated by T-helper cells.[3] It has not yet been made definitely clear which of these two types of response dominates in the pathogenesis of IPF, but it is beyond any doubt that they contribute a major role to it.[4]

Cytokines such as interferon-γ (IFN-γ) and interleukin-12 (IL-12) are essential in the development of Th-1 immune responses, while IL-4, IL-10 and the p40 subunit of IL-12 are of major significance in Th-2 immune responses.[3] IL-8 is the major neutrophil chemotactic factor of the lung.[5] IL-2 is an important inflammatory cytokine. In that concept, the above cytokines present an increased interest in the understanding of the pathogenesis of IPF.

Furthermore, as suggested already by researchers of the present study, cytokine tissue and serum levels could be of value in the enrollment of patients participating in clinical trials with immunomodulatory agents, such as IFN-γ-1β.[6] Having been studied mainly in the bronchoalveolar lavage fluid (BALF) and tissues of patients with interstitial lung disease (ILD), an insufficient amount of data exist concerning their serum levels (apart from those of IL-8) and their clinical significance in such patients.

The aim of this present study is therefore to focus on the levels of the above-mentioned cytokines, both fibrogenic and nonfibrogenic, in the serum of patients with IPF, compared to those of healthy controls (HC). Working with patients with IPF being referred to the researchers' Department from around all Central Greece, researchers of the present study aim to detect preliminary data for future research concerning the clinical use of cytokines in IPF.

Materials and methods

A prospective study was conducted from 12/1999 to 8/2001 in the Pulmonary Department of the University Hospital of Thessalia, where all patients with ILD from Central Greece are referred. Among those patients selected to participate in the study were only those who fulfilled strict clinical criteria.

Methodology: The methodology pattern was as follows: Two groups were formed. *Group 1* consisted of 20 patients with IPF, who fulfilled the clinical criteria described in the ATS/ERS consensus.[7] *Group 2* consisted of 40 healthy volunteers of adjusted age and sex (Table 1). Persons in both groups did not receive any medication at the time of the diagnosis.

Patients under treatment and those who were suffering from other systematic diseases were excluded. An informed consent was obtained from all individuals that finally participated in the present study. Data regarding patients were selected following the establishment of the diagnosis,

Table 1 Summary of the characteristics of the studied individuals.

	Mean ($\pm$SD)	
	Idiopathic pulmonary fibrosis[20]	Controls[40]
Females/males	11/9	20/20
Age (years)	72 ($\pm$7.26)	67 ($\pm$10.50)
Nonsmokers/smokers	11/9	23/17
Clubbing (yes/no)	15/5	No
Duration of symptoms (months)	24 ($\pm$3.40)	—
Dyspnea		
Grades 1–4 (median)	3.00 ($\pm$0.76)	—
FEV$_1$%	78.00 ($\pm$25.06)	90.50 ($\pm$10.00)
FVC%	61.80 ($\pm$22.56)	92.00 ($\pm$7.00)
FEV$_1$%/FVC%	0.92 ($\pm$0.21)	0.97 ($\pm$0.7)
Dlco/Va (%)	64.75 ($\pm$21.21)	81.75 ($\pm$23.90)
PO_2 (mmHg)	70.00 ($\pm$19.13)	—
PCO_2 (mmHg)	37.80 ($\pm$4.93)	—

so as to form a reliable clinical, serological and HRCT profile at the time of the diagnosis.

Technique: dyspnea was scored in four grades: 1 = exercise dyspnea, 2 = walking dyspnea, 3 = common activity dyspnea and 4 = resting dyspnea. Blood samples were taken under sterile conditions, serum was collected and then stored at −80 °C. Hemolysed specimens were excluded and a second sample was recollected. Two cytokine measurements were performed for every sample and the mean value of the two assessments was included in the statistical analysis.

Interleukins

Interleukins IL-2, IL-8, IL-4, IL-10, IL-12 (p40 subunit) and IFN-γ were measured by an enzyme-linked immunoabsorbent assay (ELISA) using Biosource ELISA kits, according to the manufacturer's guidelines. ELISA is a sensitive and specific method in the assessment of cytokine levels in the serum.[8]

Statistical analysis

Data are expressed as median and range. Comparisons between the two groups were performed using the SPSS 8 for Windows Statistical program. A multivariate analysis was first performed in order to detect independent correlations between values. Important correlations were then determined by Spearman's correlation (nonparametrical values). Correlations at a statistical high level (*P*) of less than 0.05 were regarded as statistically significant.[9,10]

Results

Description of the sample: The present sample consists of patients coming from the main agricultural area in Greece and among them all the socioeconomic classes have been represented. Smoking habits were equal between the two groups. Patients with IPF had a long duration of symptoms before proceeding to seek for medical help (mean: 24 months, standard deviation (SD): 3.4 months), dyspnea of a mean grade of 3 (SD: 0.76) and functional respiratory tests compatible with a restrictive type of disorder (Table 1). Blood gases were impaired in patients with IPF (Table 1). All patients had fine crackles ("Velcro" type) in auscultation, and clubbing was present in 15/20 patients. All of the patients suffered from progressive dyspnea on exertion. Coughing was present in 14/20 patients, dry in 10/20 and productive in 4/

20. Haemoptysis was present in 2/20 patients. Right ventricular function was not impaired at the time of the evaluation (based on echocardiogram (ECG) performed in all of the patients, and cardiac ultrasound performed in 15/20 patients).

Forty healthy volunteers made their blood samples available in that period of time. They were aged 67 years old (SD: 10.5 years) and 20/40 were males. In them, as well, all the socioeconomic classes were represented. At the time of the collection of the samples they were healthy as assessed by physical examination, chest X-ray, ECG and spirometry. They, as well, were not receiving any medication at the time of the sample collections.

IL-8, IL-2, IL-10 and IL-12 were elevated in the IPF group. IFN-γ was decreased in the IPF group compared to the HC. IL-4 and IL-8 correlated to the spirometry values, such as fast expiratory volume in 1 s (FEV$_1$%) and forced vital capacity (FVC%). IL-4 positively correlated to these values, while IL-8 did so inversely.

IL-10 was elevated in the IPF group (median = 7.085 pg/ml, range: 25.00 pg/ml), compared to the control group (median: 0.70 pg/ml, range: 2.59 pg/ml) (*P* = 0.022). The mean value of *IL-8* was significantly higher in the IPF group (median: 12.00, range: 17.42 pg/ml) than in the HC group (median: 4.50 pg/ml, range: 24.00 pg/ml) (*P* = 0.001). *IL-2* was significantly higher in the IPF group (median: 0.50 pg/ml, range: 3.00 pg/ml) than in the HC group (median: 0.05 pg/ml, range: 0.06 pg/ml) (*P* < 0.001). *IL-4* serum levels were equal between the IPF group (median: 7.25, range: 22.00 pg/ml) and the HC group (median: 7.22 pg/ml, range: 7.15 pg/ml) (*P* = 0.41). *IL-12* was significantly higher in the IPF group (median: 28.37 pg/ml, range: 202.38 pg/ml) than the HC group (median: 4.10 pg/ml, range: 5.30 pg/ml) (*P* = 0.004) (Boxplot 1).
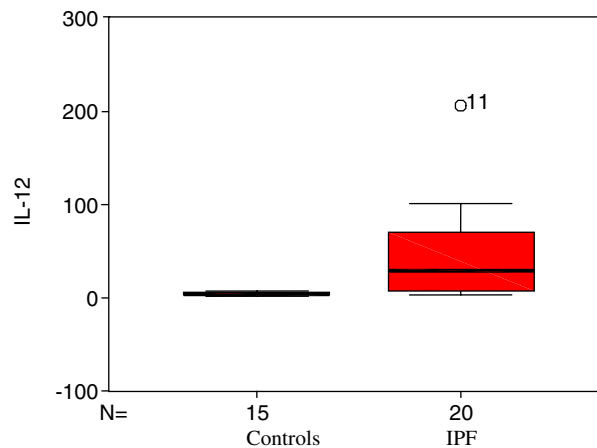
Boxplot 1 IL-12 values for patients with IPF and controls.

IFN-γ was significantly lower in the IPF group (median: 0.08 pg/ml, range: 0.80 pg/ml) than the volunteers' group (median: 0.49 pg/ml, range: 0.11 pg/ml) ($P<0.001$) (Boxplot 2, Table 2).

IL-4 positively correlated to FEV_1% ($R = 0.69, P = 0.001$) and FVC% ($R = 0.56, P = 0.012$). IL-8 negatively correlated to FEV_1% ($R = -0.58, P = 0.008$) and FVC% ($R = -0.47, P = 0.04$) (Scatterdiagrams 1 and 2).

Discussion

Th-1 and Th-2 immune responses are crucial in the development of lung fibrosis. Several studies attempt to recognize the dominant type of immune response in ILDs, among which, also in IPF.[4] However, this issue has not been made yet clear. It was long thought that Th-2 type of immunity, to an unknown yet trigger, dominates in IPF and that a change in that type of immune response towards the more favorable Th-1 response, through immu-

nomodulators such as IFN-γ-1β, would prove beneficial for the clinical outcome of those patients.[2] Unfortunately, neither the Th-2 dominance, nor the therapeutic role of Th-1 response have been completely established.

However, the presence of cytokines in the serum of patients with IPF, participating in either of the types of immune response, is of major interest both in clinical as well as research terms. The present study aims to evaluate the presence of significant cytokines of either of the types of immune response, in the sera of patients with IPF, in order to trigger further research of those presenting particular interest. The first approach to the study of cytokines in the serum is the identification of the major differences between their serum levels in patients with IPF and the respective levels in the sera of healthy volunteers.

Nevertheless, two important points must be underlined: First, although the patients were assessed at a ''common'' moment, that is the time the diagnosis was established, they cannot be expected to be in the exact same phase of the
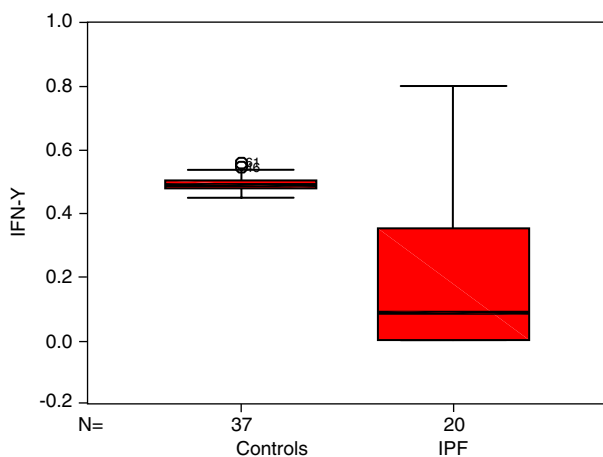

Boxplot 2 Distribution of values of IFN-γ in patients and controls.
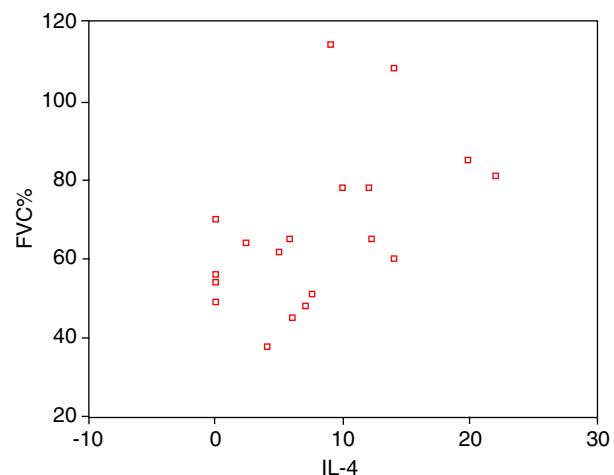
Scatterdiagram 1 Correlations between IL-4 and FVC%.

Table 2 Biochemical serum characteristics of patients with idiopathic pulmonary fibrosis (IPF) (median±range) and controls.

	Median (±range)		P
	Idiopathic pulmonary fibrosis	Controls	
IL-2 (U/ml)	0.5 (±3.00)	0.05 (±0.06)	<0.001
IL-8 (pg/ml)	12.00 (±17.42)	4.5 (±24.00)	0.001
IL-4 (pg/ml)	7.25 (±22.00)	7.22 (±7.15)	0.41
IL-10 (pg/ml)	6.50 (±25.00)	0.70 (±2.59)	0.022
IL-12 (pg/ml)	28.38 (±202.38)	4.10 (±5.3)	0.004
IFN-γ (pg/ml)	0.09 (±0.80)	0.49 (±0.11)	<0.001
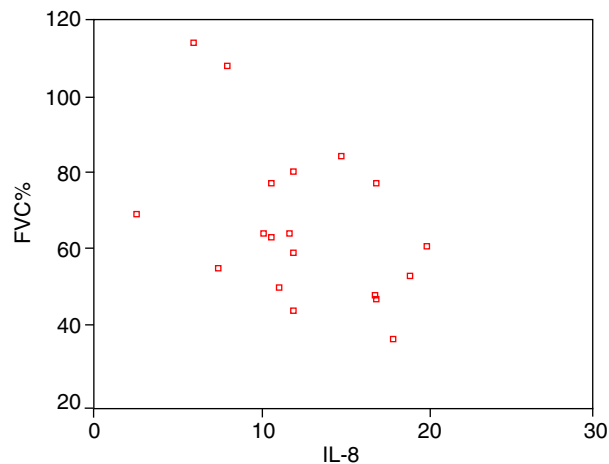
Scatterdiagram 2 Correlations between IL-8 and FVC%.

disease. Therefore, the cytokine levels represent a useful means of assessing the disease in general, not a particular phase of it.

Second, it must be strongly underlined that IPF is an organ-specific disorder, while cytokine levels were assessed in the serum. It is important to point out that serum levels of a particular cytokine do not necessarily represent the respective tissue ones. However, if these distal levels prove to be useful in assessing the disease, this information is important because they are more easily evaluated and might represent a realistic way to monitor such patients. Herein follows a discussion of each particular cytokine studied, as well as the importance of the results already provided.

IL-2 was increased in the patients' sera as compared to that of the HC. IL-2 participates in the pathogenesis of fibrosis, as it is the first inflammatory cytokine to be excreted in any immune response.[3] IL-2 participates in the immunity of T-memory cells. Its soluble receptor is detected in elevated values in the serum of patients with active ILD.[11] It correlates to the amount of inflammatory lesions in the lung.[12] IL-2 is mostly used as a serum marker in sarcoidosis.[13,14] However, it is not important as a specific marker for any ILD.[14] Its increased levels in the sera of patients with IPF should therefore be considered with caution in the clinical setting. However, it might be a cytokine worth considering in future studies of the pathogenesis of IPF, or in the enrollment of patients with IPF in clinical trials.

IL-10 is specifically elevated in the sera of patients with IPF.[15,16] As mentioned already by the researchers of the present study, serum levels of IL-10 were not detected in the sera of patients with pulmonary fibrosis of a secondary cause, while being elevated in patients with IPF.[15] IL-10 partici-

pates in Th-2 immunity. It is generally regarded as an anti-inflammatory IL, produced by alveolar macrophages (AMs); in IPF the products of the gene of IL-10 are increased in order to control the proinflammatory action of tumor necrosis factor alpha (TNF-α).[17,18] Paradoxically, its levels in the BALF of IPF patients have not been found to be high.[18] In the bleomycin-induced model of lung fibrosis, both "promoter" cytokines such as TNF-α, TNF-β, IFN-γ and IL-2, as well as a "suppressive" cytokine, such as IL-10, were found to be elevated in the fibrotic lung.[19] In another study, the levels of IL-10 in the tissues of patients with IPF were not elevated compared to those of HC.[20]

To the researchers' knowledge, its levels in the serum of patients with IPF have not been studied before. Given the evolving data suggesting it is involved in the pathogenesis of IPF, and the researchers' failure to detect any IL-10 levels in patients with other ILDs, it might be suggested that further research regarding IL-10's possible role in the pathogenesis and treatment of patients with IPF might be interesting. Given the failure of patients with IPF to respond to corticosteroids, while patients with ILD due to other causes benefit from them, as well as the different IL-10 serum status in those patients groups, it might be suggested that IL-10 might be a cytokine especially involved in IPF, and therefore of evolving significance for future research.

However, once again, the serum levels do not necessarily represent the tissue ones. Therefore, the elevated levels of IL-10 in the sera of patients with IPF might just represent the lack of effect of IL-10 in suppressing the inflammatory response in IPF. IL-10 might be stimulated by other cytokines, as part of a Th-2 response and therefore might be a less significant cytokine in IPF. Those two contradictory hypotheses might be elucidated when more data from future research are made available.

IL-8 is the most well-studied cytokine in patients with fibrosis.[5] IL-8 has been found to be elevated in the BALF and serum of patients with IPF and to reflect disease activity.[21] It is released by activated mononuclear macrophages and has a potent chemotactic activity for polymorphonuclear leukocytes. Therefore, it contributes to the amount of neutrophilic alveolitis in IPF. It is elevated in the BALF cells of patients with IPF and sarcoidosis, though it is not clear if it corresponds with the BALF neutrophilia or not.[22,23] It seems probable that IL-8 is increased in the BALF of patients with IPF during the subacutely progressive phase rather than the chronic phase of the disease.[24]

In accordance with the available data, in the present study IL-8 was elevated in patients with IPF

and negatively correlated to FEV_1% and FVC%. This result could be interpreted as IL-8 being a serum marker of spirometry impairment in patients with IPF. However, since IL-8 has been fairly studied in IPF and, furthermore, current concepts about the pathogenesis of IPF suggest that the role of neutrophil inflammation and alveolitis in it is of minor importance, IL-8 seems to be less involved in the pathogenesis of IPF than previously thought.[25] However, its clinical value should not be underestimated, since it is clearly a cytokine the serum levels of which represent physiological impairment in IPF.[21]

IL-4 has not been previously studied in the sera of patients with IPF. IL-4 is a cytokine participating in the development of the Th-2 response.[4] It is a fibrogenic cytokine, secreted by cells such as T-lymphocytes and mast cells, that increases collagen production by fibroblasts.[26] Greater numbers of IL-4 positive cells in IPF tissues are found at the advanced stage of the disease, and augmented amounts of IL-4 have been reported in situations such as radiation-induced pneumonitis in rats.[27,28] IL-4 correlated positively to the spirometry values of FEV_1% and FVC%. Therefore, it correlates with a less severe restrictive disorder. However, its serum levels in patients with IPF are among the normal values, as assessed by the HC group. This present study does not suggest that serum measurements of IL-4 are of major interest in the clinical setting of patients with IPF.

IFN-γ was decreased in the sera of patients with IPF. IFN-γ derives by T-lymphocytes and mast cells and decreases collagen production by fibroblasts.[29] It is a major cytokine of the Th-1 immune response.[3] IFN-γ is considered to have an antifibrotic effect based on the down-regulation of the bleomycin-induced overexpression of transforming growth factor-beta (TGF-β) and subsequently procollagen m-RNAs, leading to a decreased collagen content.[30] The pathogenesis of fibrosis partly depends on the imbalance between cytokines derived by type II alveolar epithelial cells, such as IL-4 and IFN-γ with contradictory effects, which also reflect a Th-1–Th-2 imbalance, respectively.[31]

IFN-γ has already been found to be decreased in the fibrotic tissue.[32] It has also been shown that patients with IPF who have high levels of IFN-γ in their serum respond better to corticosteroids.[32] In an effort to convert the Th-2 environment in the field of fibrosis, IFN-γ-1β has been tested as a therapeutic agent in patients with IPF.[33] The results of a preliminary study showed that patients with IPF who had reduced amounts of IFN-γ in their tissues, benefited from therapy with the above-mentioned agent.[33] However, a large randomized

multicentered trial, in which tissue levels of IFN-γ were not assessed, proved that IFN-γ-1β did not provide any important improvement in those patients.[34] The question remains however, if IFN-γ-1β would have been beneficial for a subgroup of patients having reduced tissue IFN-γ.

It is therefore agreed that IFN-γ is too important an agent in IPF to be abandoned in future research.[35] Assessment of IFN-γ serum levels in patients with IPF of the present study proved that they have inadequate circulating IFN-γ. Further correlations between tissue and serum levels of IFN-γ in patients with IPF are needed, in order to selectively enroll patients in future trials with IFN-γ-1β. Clearly, future studies are needed to elucidate its role in this devastating disease.

IL-12 derives principally by activated macrophages. Little data exist about the role of IL-12 in lung fibrosis. Existing studies are mostly made in mice, and prove that IL-12 plays a certain role in the pathogenesis of lung fibrosis by regulating the T-helper type-1 cell action.[36] IL-12 is comprised by two separate subunits: p40 and p35 which form the p70, that corresponds to IL-12.[37] While p35 subunit might have an antifibrotic effect, the p40 subunit participates in T-helper 2-cell polarization, which plays an important role in fibrosis due to inhaled inorganic particles.[38] IL-12 (p40 subunit) was elevated in the sera of IPF patients of the present study. It seems to be an interesting cytokine to study, since the clarification of its role in the pathogenesis of fibrosis might lead to new therapeutic approaches.[39] Results of the present study suggest it is worth studying in the clinical setting, as well.

Cytokines are considered to play a significant role in lung injury and repair and are important in the pathogenesis of fibrosis. Some of them seem to participate in the local injury and inflammatory reaction (IL-1, IL-8, TNF-alpha), while some others are involved in tissue repair and fibrosis (PDGF, IGF-1, TGF-β). Cytokines form complex networks, orchestrated by key cytokines, and the most important feature in understanding the pathogenesis of the fibrosis seems to be the balance of positive (profibrogenic) and negative (antifibrogenic) forces generating from interactions between the various cytokines.[40] In those complex networks, cytokines regulate the fibroblast function and fibroblasts feedback to regulate the inflammatory function. A given IL, however, has variable effects depending on the activation of the target cell, the local presence of other cytokines and the ability of the target cell to produce bioactive autocoids, such as prostaglandins. Cytokines in combination have different effects on fibroblast proliferation than

they have individually.[41] This is therefore one of the main limitations in the study of such molecules.

Cytokines involved in lung fibrosis have been rather adequately studied in the BALF of patients with IPF or other interstitial fibrosis, and the local action at the place of inflammation and fibrosis is becoming more evident. However, apart from IL-8, there are not enough data considering the values of the ILs in the serum of patients with IPF.

In conclusion, the present study of serum cytokines in IPF suggests that IFN-γ, participating in Th-1 immune response was decreased in the sera of the studied patients with IPF. IL-4, participating in Th-2 response was within the normal values in the sera of patients with IPF. On the other hand, IL-2 (a purely inflammatory cytokine), IL-10 and IL-12-p40 subunit (participating in Th-2 immunity), as well as IL-8, which is a chemoattractant cytokine, were all increased in the sera of the studied patients with IPF. The Th-2 predominance, which is considered to be one of the main, if not the major pathogenetic factor in IPF is suggested, though not definitely, also by results of the present study. The present report aims to provide preliminary data in order to induce future research in the field of cytokines and IPF, either in the serum or in the tissues of such patients. Hopefully, the exploration of their clinical significance and possible role in the pathogenesis of IPF would lead to new therapeutic paths.

Acknowledgments

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