

Simultaneous measurement of collagen type-VI-related antigen and procollagen type-III-N-propeptide levels in bronchoalveolar lavage

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Simultaneous measurement of collagen type-VI-related antigen and procollagen type-III-N-propeptide levels in bronchoalveolar lavage. T. Schaberg, K. Orzechowski, C. Oesterling, H. Lode, D. Schuppan. ©ERS Journals 1994.

ABSTRACT: Abnormal collagen metabolism is a hallmark of diffuse lung disease. Biochemical parameters which are correlated with collagen synthesis and degradation may be helpful to monitor fibrosis. In this study, we compared the sensitivity and specificity of procollagen type-III-N-propeptide (PIIINP) and collagen type-VI (C-VI) related antigen levels, as well as a ratio of both parameters (PIIINP/C-VI), in bronchoalveolar lavage fluid (BALF) from patients with diffuse and localized lung disease.

We investigated 45 patients with diffuse lung disease (idiopathic pulmonary fibrosis (IPF), n=21; sarcoidosis, n=13; and lymphangitic carcinomatosis (LC), n=11); 58 control subjects; and 92 patients with localized lung disease (bronchial carcinoma, n=37; pulmonary tuberculosis, n=31; and pneumonia, n=24). C-VI and PIIINP were measured by immunoassay in concentrated BALF.

Although the PIIINP and C-VI levels were increased in diffuse lung disease, the sensitivity of the individual parameters PIIINP and C-VI was low (IPF: PIIINP=0.62, C-VI=0.29; LC: PIIINP=0.64, C-VI=0.45; sarcoidosis: PIIINP=0.69, C-VI=0.15). When calculating the ratio of PIIINP/C-VI for each individual patient, we found a significant increase in this ratio in IPF (1.28±0.7), LC (2.34±1.2), and sarcoidosis (0.26±0.08) compared to both the controls (0.02±0.01) and other localized lung diseases (bronchial carcinoma 0.05±0.01; pulmonary tuberculosis 0.02±0.01), with the exception of pneumonia (0.18±0.06). A ratio for PIIINP/C-VI ≥0.03 was sufficiently specific (specificity in controls 0.88; in tuberculosis 0.87; and in bronchial cancer 0.79) and more sensitive (sensitivity in IPF 0.86; in sarcoidosis 0.85; and in LC 0.82) than each individual parameter.

Simultaneous measurement of C-VI and PIIINP in BALF from patients with diffuse chronic lung diseases may be a more sensitive approach to assess disturbances in collagen metabolism than the determination of each single parameter.

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Pulmonary fibrosis is a common final pathway in numerous inflammatory and immunological lung diseases that display a roentgenographic picture of diffuse infiltration [1]. A hallmark of these diseases is an abnormal metabolism of connective tissue polypeptides [2]. However, clinical and roentgenographic data, as well as pulmonary function tests, are frequently only modestly correlated with the degree of pulmonary fibrosis. Thus, biochemical measurements of the balance between collagen synthesis and degradation would be helpful in monitoring the progress of fibrotic lung disease. In recent years, an assay for the procollagen type-III N-terminal propeptide (PIIINP) [3, 4] has been used to assess collagen turnover both in serum and bronchoalveolar lavage fluid (BALF) from patients with idiopathic pulmonary fibrosis [5, 6], sarcoidosis [7, 8], adult respiratory distress syndrome [9, 10], and farmer's lung

[11–13]. Most of these studies have investigated PIIINP levels either in serum or BALF: In a few studies both PIIINP levels were investigated [10, 14, 15]. However, none of these studies considered other parameters of collagen metabolism in BALF.

Measuring only a single parameter of collagen metabolism presents a serious disadvantage, since no valid method of determining the concentration of soluble substances in the BALF of patients with diffuse lung diseases is available. Therefore, values from patients and healthy subjects are difficult to compare [16]. Measuring two parameters of collagen metabolism simultaneously in BALF may be more powerful than either parameter alone, especially when using one parameter related to collagen synthesis, and another one to collagen degradation. Furthermore, by calculating the ratio of two parameters the result is independent of either specific concentration.

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We chose PIIINP to reflect collagen production, since a significant proportion of PIIINP is produced by cleavage of procollagen peptides from the N-terminal end of this molecule during fibril formation [17]. As a parameter of collagen degradation, we chose collagen type-VI (C-VI)-related antigen, which anchors the large collagen fibrils to each other [17], and in contrast to PIIINP, does not undergo extracellular processing [18]. The antigen used for the C-VI assay was isolated from pepsin-digested human placenta. Gel electrophoresis under reducing conditions revealed three prominent bands of 43, 50 and 55 kD in molecular weight, respectively, representing three distinct polypeptide chains [19]. Using an assay based on this non-crossreacting antigen, we have shown that in fibrotic liver disease collagen degradation was responsible for circulating C-VI in serum and other body fluids [20].

We hypothesized that the ratio of C-VI and PIIINP levels (C-VI/PIIINP) in BALF of patients with diffuse lung diseases would be a more sensitive test to detect abnormalities than the determination of either variable alone. We reasoned that the ratio would be independent of a denominator substance in BALF. To determine whether or not our measurements are specific for diffuse lung disease, we used BALF samples not only from healthy controls, but also from patients with a variety of localized lung diseases for comparison.

Material and methods

Study population

One hundred and ninety five patients were entered into the study between 1991 and 1993. The patients were classified as belonging to one of the following seven groups: 1) patients with a newly diagnosed idiopathic pulmonary fibrosis (IPF), n=21; 2) patients with a newly diagnosed pulmonary sarcoidosis stage II (S), n=13; 3) patients with a newly diagnosed lymphangitic carcinomatosis (LC), n=11; 4) patients with bronchial cancer, n=37; 5) patients with bacterial pneumonia, n=24; 6) patients with pulmonary tuberculosis, n= 31; and 7) control subjects who were adults without any acute pulmonary disease, n=58.

All diagnosis of IPF, LC and sarcoidosis were based on histological findings from specimens obtained by bronchoscopy, thoracoscopy or surgical open lung biopsy, supported by a compatible history, pulmonary function tests, and laboratory values. In patients with pneumonia or tuberculosis, the diagnosis was based on culture of a relevant micro-organism, and clinical evidence. For patients with lung cancer, the diagnosis was based on histological or cytological specimens obtained by bronchoscopy. Pulmonary function tests in patients with diffuse lung disease revealed reduced lung volumes and a marked impairment of gas exchange in patients with IPF (vital capacity (VC) $54\pm 27\%$ predicted; total lung capacity (TLC) $68\pm 22\%$ pred; transfer coefficient (TL/VA) $52\pm 17\%$ pred; arterial oxygen tension (P_{aO_2}) 8.7 ± 3.2 kPa (65 ± 24 mmHg)) and LC (VC $66\pm 17\%$

pred; P_{aO_2} 8.8 ± 2.1 kPa (66 ± 16 mmHg)) and a mild impairment only of gas exchange for patients with sarcoidosis (VC $84\pm 10\%$, TLC $81\pm 10\%$, TL/VA $71\pm 11\%$ pred; P_{aO_2} 10.4 ± 1.6 kPa (78 ± 12 mmHg)).

Bronchoalveolar lavage (BAL) of control subjects was performed during bronchoscopy in the course of diagnostic procedures performed for a history of previously observed minor haemoptysis, a suspected localized airway stenosis, a suspected hilar or mediastinal mass, or other minor findings on the chest X-ray. In all of these patients, intensive diagnostic procedures did not reveal any acute pulmonary disease. Smoking history was determined as described [21].

Signed informed consent according to the Helsinki Declaration was obtained from each patient. The study was approved by the Local Ethics Committee. None of the patients was receiving corticosteroids or cytotoxic drugs at the time of investigation. Smokers stopped at least 12 h before the BAL.

Bronchoalveolar lavage

BAL was performed *via* a fiberoptic bronchoscope (FBF 5, Olympus, Tokyo, Japan) using 160 ml of sterile 0.9% saline at 30°C in 20 ml aliquots, as described previously [21]. In all cases with bronchial cancer and in the controls, we performed BAL on the contralateral side (middle lobe or lingula) of the radiological findings. In patients with tuberculosis or pneumonia, the segments involved were lavaged. All BAL procedures were performed before biopsies in order to avoid contamination by blood.

Lavage fluid specimens were filtered through sterile gauze and centrifuged at $500\times g$ for 8 min at 4°C. The cells were separated, quantified and identified, as described previously [21], and used for other studies. BALF supernatants were immediately frozen and stored at -80°C until further concentration.

Concentration of BALF

Prior to concentration, bovine serum albumin (BSA), (Sigma, Deisenhofen, FRG) was added to the lavage fluid to yield a final concentration of 5 mg in 100 ml, followed by centrifugation at 5,000 rpm for 10 min at 4°C to remove insoluble substances. The supernatants were dialysed (size exclusion 12 kD) exhaustively against four changes (every 8 h) of 0.05% acetic acid at 4°C.

The dialysed lavage was lyophilized and the lyophilisate dissolved in a volume of phosphate buffered saline (PBS) (Sigma) to yield a concentration factor of 50. Alternatively, a minor fraction of lavage supernatants was subjected to two rounds of ultrafiltration (Diaflow-ultrafiltration cartridges and Centricon, size exclusion limit 10 kD, Amicon, Witten, FRG) to obtain a 50–60 fold concentration. Concentration experiments using both methods for a single sample showed comparable data for PIIINP and C-VI within the limits of assay variability (usually below 10%).

Measurement of collagen type-VI-related antigens

C-VI was isolated from pepsin-digested human placenta by extraction in acetic acid, fractional salt precipitation in acidic and neutral buffers, followed by agarose gel chromatography on Agarose A-1.5M (Bio-Rad, Munich, FRG). Preparations of C-VI were at least 95% pure, as assessed by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), rotary shadowing electron microscopy and radioimmunoassay, as described previously [19]. Specific antibodies were produced in New Zealand White rabbits after primary immunization with 0.3–0.5 mg of antigen [19]. Specificity of the antibody was demonstrated by radioimmuno-binding assay with ¹²⁵I-labelled C-VI and by Western blotting [22].

C-VI was quantified by a newly developed two-armed enzyme-linked immunosorbent assay (ELISA) system [22, 23] based on competition of serial dilutions of the standard or the sample with a predetermined amount of rabbit antibodies to human placental C-VI. Uncompeted antibodies were then bound to C-VI immobilized on a 96-well microtitre plate and quantified using biotinylated goat anti-rabbit immunoglobulin G (IgG), followed by rabbit peroxidase antiperoxidase and extravidin-peroxidase. Inter- and intra-assay variability were below 10%. C-VI levels are expressed as ng·ml⁻¹ unconcentrated lavage.

Measurement of PIIINP-related antigens

PIIINP was determined using a commercially available test kit (PIIIP-Riagnost, Behringwerke, Marburg, FRG). This assay measured PIIINP-related antigens with a sensitivity of 1 ng·ml⁻¹ (0.1 E·ml⁻¹) [24] in concentrated BALF. Inter- and intra-assay variability for concentrated lavage fluids were below 10 and 5%, respectively. PIIINP levels are expressed as ng·ml⁻¹ unconcentrated lavage.

Statistical analysis

Statistical analysis was carried out on a Macintosh LC-computer (Apple Inc., Cupertino, CA, USA) using a commercial statistics program (InStat, Instant Biostatistics, GraphPad , San Diego, CA, USA). Values were expressed as mean±SEM, median values and range. Differences in median values were analysed using the Mann-Whitney-Wilcoxon U-test. Because of the multiple comparisons, an adjustment was made to control for error rates in multiple U-tests. Differences in median values of the ratio PIIINP/C-VI were analysed using Kruskal-Wallis nonparametric analysis of variance (ANOVA) test. Because of the multiple comparisons, an adjustment was made to control for error rates in multiple tests using Dunn's multiple comparisons test. P-values ≤0.001 were considered to be significant. Sensitivity was determined as: true positive test results/(true positive test results+false negative test results); and specificity was determined as: true negative test results/(true negative test results+false positive test results). To determine sensitivity and specificity we used the 95th percentile of control subjects as normal values for the PIIINP assay (<0.06 ng·ml⁻¹ BALF) and for the C-VI assay (<1.72 ng·ml⁻¹ BALF). For each individual subject, we calculated the value of the ratio PIIINP/C-VI. To test differences of the parameter PIIINP/C-VI <0.03 or ≥0.03 between controls and fibrotic lung diseases we used 2×2 contingency tables and the χ^2 -test (Yates corrected) with $\chi^2 >10.82$ (p<0.001).

Results

BAL data

The clinical and BAL data are displayed in table 1. Fluid recovery in all groups was similar, as was the total number of cells. As expected, the BAL differential cell

Table 1. – Basic clinical and BAL data

| Diagnosis | Age yrs | Smoker/ nonsmoker n | BAL recovery % | Total cells per ml BALF ×10 ⁴ | AM % | LYM % | PMN % |
|----------------------------|------------|---------------------------|----------------------|--|---------|----------|----------|
| Control (n=58) | 52±14 | 28/30 | 63±6 | 14.9±1.2 | 92±1 | 5±2 | 3±1 |
| Bronchial cancer (n=37) | 61±10 | 29/8 | 57±15 | 26.3±7.1 | 94±1 | 3±1 | 3±1 |
| Tuberculosis (n=31) | 39±11 | 22/9 | 53±22 | 13.3±2.7 | 93±2 | 5±3 | 2±1 |
| Pneumonia (n=24) | 44±14 | 12/12 | 54±17 | 18.7±6.3 | 72±8 | 3±2 | 25±11 |
| IPF (n=21) | 59±16 | 7/14 | 62±20 | 19.8±3.1 | 80±3 | 9±3 | 11±2 |
| Sarcoidosis (n=13) | 39±11 | 2/11 | 63±14 | 14.2±2.1 | 76±11 | 21±8 | 3±2 |
| LC (n=11) | 53±10 | 6/5 | 54±11 | 15.2±4.7 | 89±4 | 5±3 | 6±3 |

Values are presented as mean±SEM. AM: alveolar macrophages; LYM: lymphocytes; PMN: polymorphonuclear granulocytes; IPF: idiopathic pulmonary fibrosis; LC: lymphangitic carcinomatosis; BAL: bronchoalveolar lavage; BALF: BAL fluid.

counts were different between the groups, showing a lymphocytosis in patients with sarcoidosis, an increased number of PMN and lymphocytes in IPF patients, and an increase in PMN in patients with pneumonia. No significant correlation was found between the mean age, sex, smoking status, or BAL cellularity and the results of the two assays used in the study.

Collagen type-VI-related antigen

Antigens related to C-VI were detected in BALF of all patients. In IPF (median 1.38 ng·ml⁻¹ BALF) and LC (median 1.20 ng·ml⁻¹ BALF) the values were significantly ($p < 0.001$) increased compared to controls, but not in patients with sarcoidosis (median 1.14 ng·ml⁻¹ BALF) (table 2).

For control subjects, the median value was lower (0.75 ng·ml⁻¹ BALF) within a range of 0.14–2.78 ng·ml⁻¹ BALF, and the 95th percentile at 1.72 ng·ml⁻¹ BALF. Similar low median values were obtained for patients with pneumonia (1.02 ng·ml⁻¹ BALF), bronchial cancer (0.98 ng·ml⁻¹ BALF), and tuberculosis (0.96 ng·ml⁻¹ BALF) (table 2).

For the C-VI assay the sensitivity of a value ≥ 1.72 ng·ml⁻¹ (95th percentile of controls) was 0.45 in LC, 0.29 in IPF and 0.15 in sarcoidosis, and the specificity based on control values was 0.86.

Procollagen type-III-N-propeptide

For control subjects, the median values of PIIINP were below 0.01 ng·ml⁻¹ BALF, and the 95th percentile was ≤ 0.06 ng·ml⁻¹. In patients with lymphangitic carcinomatosis, we obtained the highest median values of PIIINP (0.90 ng·ml⁻¹ BALF), followed by patients with

Table 2. – Collagen type-VI-related antigen and procollagen type-III-N-propeptide levels in BALF

| Diagnosis | C-VI-related antigen ng·ml ⁻¹ BALF | PIIINP ng·ml ⁻¹ BALF |
|------------------|--|------------------------------------|
| Control | 0.75 (0.14–2.78) | ≤ 0.01 (0.01–0.24) |
| Bronchial cancer | 0.98 (0.40–2.60) | ≤ 0.01 (< 0.01 –0.78) |
| Tuberculosis | 0.96 (0.50–3.60) | ≤ 0.01 (< 0.01 –0.19) |
| Pneumonia | 1.02 (0.17–1.60) | 0.03 (< 0.01 –0.65) |
| IPF | 1.38* (0.80–5.57) | 0.11* (< 0.01 –11.5) |
| Sarcoidosis | 1.14 (0.54–2.22) | 0.20* (< 0.01 –0.67) |
| LC | 1.20* (0.82–4.27) | 0.90* (< 0.01 –15.3) |

Values are presented as median, and range in parenthesis. C-VI: collagen type-VI; PIIINP: procollagen type-III-N-propeptide. For further abbreviations see legend to table 1. *: values significantly different from controls ($p < 0.001$).

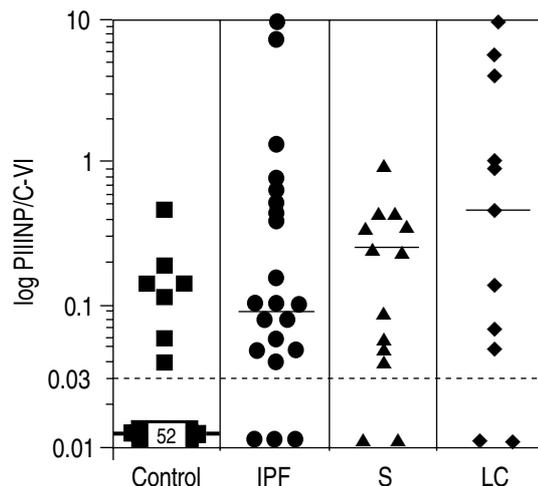


Fig. 1. – Values of the individual ratio of PIIINP/C-VI in BALF samples from patients with diffuse lung disease. ■ : controls; ● : patients with idiopathic pulmonary fibrosis (IPF); ▲ : sarcoidosis (S); ◆ : lymphangitic carcinomatosis (LC). The dashed line shows the upper limit of normal values. The black lines represents the median values which for IPF, S and LC differ significantly from the controls ($p < 0.001$). PIIINP: procollagen type-III-N-propeptide; C-VI: collagen type-VI; BALF: bronchoalveolar lavage fluid.

sarcoidosis (0.20 ng·ml⁻¹ BALF) and IPF (0.11 ng·ml⁻¹ BALF) (all $p < 0.001$ compared to control). However, in all lung diseases PIIINP values displayed a relatively wide range (table 2).

Detection of PIIINP was also low for patients with bronchial cancer (10 out of 37; 27%), and tuberculosis (7 out of 31; 23%); whereas, a higher proportion of patients with pneumonia showed elevated levels (15 out of 24; 63%) (table 2).

The sensitivities of PIIINP values ≥ 0.06 ng·ml⁻¹ (95th percentile of control) obtained in diffuse lung diseases were as follows: IPF 0.62; LC 0.64; and sarcoidosis 0.69. The specificity of the PIIINP assay in controls was 0.88.

Ratio of PIIINP/C-VI

The mean values of the PIIINP/C-VI ratio for patients with IPF were 1.28 ± 0.76 ; for patients with sarcoidosis 0.26 ± 0.08 ; and for patients with LC 2.34 ± 1.23 (all $p < 0.001$ compared to the control patients) (fig. 1). The specificity for a ratio of PIIINP/C-VI < 0.03 in control subjects was 0.88; bronchial cancer 0.79; tuberculosis 0.87; and pneumonia 0.54. Calculating the sensitivity of a ratio PIIINP/C-VI ≥ 0.03 we found a value of 0.86 for IPF; 0.85 for sarcoidosis; and 0.82 for LC. Chi-squared tests of 2×2 contingency tables for a ratio < 0.03 versus a ratio ≥ 0.03 revealed χ^2 values for IPF of 35.32; for sarcoidosis of 25.81; and for LC of 21.49, when compared to control subjects (all $p < 0.001$).

In all other patients, except those with pneumonia (0.18 ± 0.06), we found low mean values of the PIIINP/C-VI ratio (bronchial cancer 0.05 ± 0.04 ; tuberculosis 0.02 ± 0.01) (table 3).

Table 3. – PIIINP/C-VI ratio in various lung diseases

| Diagnosis (n) | PIIINP/C-VI [†] ratio | PIIINP/C-VI ≥0.03 n |
|----------------------------|-----------------------------------|------------------------|
| Control (n=58) | 0.02±0.01 | 7 |
| Bronchial cancer (n=37) | 0.05±0.04 | 8 |
| Tuberculosis (n=31) | 0.02±0.01 | 4 |
| Pneumonia (n=24) | 0.18±0.06* | 11 |
| IPF (n=21) | 1.28±0.76* | 18 |
| Sarcoidosis (n=13) | 0.26±0.08* | 11 |
| LC (n=11) | 2.34±1.2* | 9 |

†: values are presented as mean±SEM. *: values significantly different from controls ($p<0.001$). For abbreviations see legends to tables 1 and 2.

Discussion

To our knowledge, this is the first report with simultaneous measurement of PIIINP and C-VI in BALF. Our measurements of PIIINP agree with previous studies indicating elevated PIIINP levels in BAL of patients with diffuse lung diseases, such as IPF, adult respiratory distress syndrome (ARDS), sarcoidosis and farmer's lung [5–8, 10–15].

Measurement of one single collagen-related parameter in BALF presents two major problems. Firstly, no denominator substance is known for standardization of solubles in BALF [16, 25]. Secondly, a single collagen metabolite does not apparently provide sufficient information about the impairment of collagen production and degradation in the lung.

To overcome the problem of a denominator substance for the assessment of concentration values in BALF, we calculated the ratio PIIINP/CV-I to obtain a value which is independent of a concentration dimension. Such standardization of BAL is a great advantage, since damage to the alveolar capillary membrane in chronic inflammatory disease may cause a leakage of serum proteins from the blood vessels into the alveolar space. This leakage cannot be precisely quantified [16]. Thus, normalization to urea, total protein, or albumin in BAL may be misleading, since the origin of these substances is unclear [25]. Furthermore, the leakage problem is inhomogeneously distributed in patients with the same disease, and may differ from region to region in the diseased lung [26].

The approach of calculating the PIIINP/C-VI ratio may provide the first step in a solution to the second problem. The deposition and removal of collagen is a multistep process, which involves intracellular synthesis of the procollagen, secretion into the extracellular space, extracellular processing by a more or less complete removal of the N- and C-propeptides, fibril formation, cross-linking of the molecules, and eventually their breakdown. Therefore, it is very difficult to

assess disorders in this process by measuring only one collagen metabolite [17, 20]. There is growing experimental evidence that PIIINP levels are primarily due to increased collagen type-III production. PIIINP reflects the cleavage of procollagen peptides from the N-terminal end of collagen type-III during fibril growth [20, 27]. However, PIIINP may also be released from pre-existing collagen fibrils, which bear incompletely processed procollagen type-III molecules on their surface, and may, therefore, under some circumstances also reflect increased collagen breakdown [17, 27, 28]. On the other hand, C-VI antigens in serum have been found to reflect degradation of C-VI [19]. Presumably, C-VI levels measured by our assay represent a degradation product derived from the characterization of the immunoreactive component as a molecule with an M_r of 100 kD [19], whereas the intact collagen type-VI molecule displays an M_r of 500 kD under nonreducing conditions [28].

So far, C-VI has been determined only in serum and urine of patients with liver disease. In chronic hepatitis, cirrhosis and hepatic schistosomiasis, C-VI serum levels were elevated, irrespective of the disease aetiology [20, 23]. In all these liver diseases, a histologically proven progress of fibrosis was correlated with an increase in serum PIIINP levels and a relative decrease of serum C-VI levels [19, 22, 23]. If the results in liver disease patients are applicable to those obtained from patients with lung disease, one could speculate that in diffuse lung disease, such as sarcoidosis and IPF, an increase of the PIIINP/C-VI ratio may indicate the supposed shift in collagen metabolism in favour of fibrosis [29]. The high values of the ratio in LC must be explained by some other mechanism. Tumour growth inside lymphatic vessels may impair the clearance of PIIINP *via* the lymphatic system [30], followed by increased values in the epithelial lining fluid. As expected, in tuberculosis and bronchial cancer no major imbalance of collagen metabolism was found, whilst in pneumonia a local impairment of collagen metabolism may occur.

Two major problems must be addressed before the determination of the PIIINP/C-VI ratio can be used for clinical purposes. The first is to evaluate the PIIINP/C-VI ratio in correlation to physiological parameters, symptoms and X-ray scores. This has been done by others for PIIINP levels in BALF by using the alveolar-arterial oxygen difference ($A-aDO_2$) [15], or by using roentgenographic methods [5]. The second problem involves disease activity. In farmer's lung and in sarcoidosis, increased concentrations of PIIINP, vitronectin or fibronectin seem to predict the ongoing inflammation and not necessarily a fibrotic process [13, 31, 32]. Therefore, patients must be investigated longitudinally before the PIIINP/C-VI ratio can be established as an improved marker for the progression and activity of fibrotic lung disease. If a correlation between PIIINP/C-VI ratio in BALF and disease activity can be documented, its determination may be of clinical value, particularly in the evaluation of drugs aimed at preventing fibrosis.

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