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## Immunocytological and mineralogical study of bronchoalveolar lavage in a group of subjects exposed to asbestos

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**RIASSUNTO.** 25 soggetti (24 maschi e 1 femmina, età media 57,4 anni), esposti ad amianto, sono stati sottoposti a: Radiografia torace, Tac torace ad alta risoluzione (HRCT), Prove funzionali e Lavaggio Broncoalveolare (BAL) con determinazione delle componenti cellulari (cellularità totale, percentuale di macrofagi, linfociti, granulociti ed eosinofili e sottopopolazioni linfocitarie CD3+, CD4+, CD8+, CD19+ e HLADR+) e solubili (IL8, IL10, IL12 e MCP-1 nel surnatante). I soggetti esaminati sono stati suddivisi in gruppi, in base all'entità dell'esposizione, alla concentrazione di fibre di asbesto nel BAL e al quadro radiologico secondo la classificazione I.L.O. (0/0, 0/1 e 1/0 e superiori). In rapporto agli indici di esposizione, è stato evidenziato un significativo aumento della percentuale di linfociti ( $p < 0.05$ ) nel BAL dei soggetti con esposizione di grado moderato e della concentrazione di IL-10 ( $p < 0.05$ ) nel surnatante dei soggetti con assenza di corpuscoli dell'asbesto nel BAL. Nel gruppo con quadro radiologico 0/0 e 0/1 si è osservata una più elevata percentuale di linfociti ( $p < 0.02$ ) nel BAL, mentre un aumento dei granulociti neutrofilici si è notato nei casi con più evidenti segni di fibrosi polmonare. La percentuale dei neutrofilici è risultata inversamente correlata ad alcuni parametri funzionali respiratori quali la capacità vitale e la pressione parziale di ossigeno nel sangue. Lo studio delle citochine, nel surnatante del BAL, ha evidenziato una riduzione della concentrazione dell'IL-10 con la progressione del quadro radiologico, ed una correlazione negativa, statisticamente significativa, di tale citochina con la  $pO_2$  ( $p = 0.048$ ). Per quanto riguarda le altre citochine e chemochine studiate (MCP-1, IL8 e IL12) non sono state rilevate differenze significative in base al quadro radiologico. Si è però evidenziata una correlazione positiva fra concentrazione di IL8 e percentuale di neutrofilici ( $p = 0.038$ ) e fra concentrazione di MCP-1 e percentuale di linfociti ( $p = 0.006$ ). È stata inoltre osservata una correlazione negativa tra le concentrazioni di IL-10 e IL-12 nel BAL ( $p = 0.028$ ). I risultati di questa ricerca inducono a ipotizzare che IL-10 eserciti un importante ruolo nella patogenesi e nella evoluzione della asbestosi.

**Parole chiave:** asbestosi, lavaggio broncoalveolare, citochine.

**ABSTRACT.** [www.gimle.fsm.it](http://www.gimle.fsm.it)

Twenty-five subjects (24 males and 1 female, mean age 57.4 years) who have been exposed to asbestos underwent chest radiography, high resolution computed tomography (HRCT) of the chest, lung function tests and bronchoalveolar lavage (BAL) for evaluation of cell components (total cell count, percentages of macrophages, lymphocytes, neutrophil and eosinophil granulocytes and the lymphocyte subpopulations CD3+, CD4+, CD8+, CD19+ and HLADR+), soluble factors (IL-8, IL-10, IL-12 and MCP-1 in the supernatant) and concentration of asbestos fibre. The subjects were subdivided according to the degree of

their exposure, to the concentration of asbestos fibres in the BAL and to chest X-ray findings using the I.L.O. classification (0/0pl, 0/1 and 1/0 and above). According to the exposure index, we showed statistically significant ( $p < 0.05$ ) higher lymphocytes percentage in the BAL of subjects with moderate exposure and significantly higher levels of IL-10 ( $p < 0.05$ ) in the supernatant of subjects showing an absence of asbestos fibres in their BAL. In the group of subjects with a 0/0 and 0/1 radiological profile, the cellular component of the BAL was characterised by a higher percentage of lymphocytes ( $p < 0.02$ ), whereas a trend toward an increase in the number of neutrophils was noted in subjects with obvious pulmonary fibrosis. The percentage of neutrophils was inversely correlated with some parameters of respiratory function such as vital capacity ( $p = 0.03$ ) and the partial pressure of oxygen ( $p = 0.05$ ) in the blood. Investigating the cytokines in the supernatant of the BAL, we found a trend toward lower concentration of IL-10 in the group showing the worst radiological picture (I.L.O.  $\geq 1/0$ ), and a statistically significant negative correlation between this cytokine and  $pO_2$  ( $p = 0.048$ ). Concerning the other cytokines and chemokines studied (MCP-1, IL-8 and IL-12), no significant differences were found to be associated with the radiological profiles. There were, however, positive correlations between the concentration of IL-8 and the percentage of neutrophils ( $p = 0.038$ ) and between the concentration of MCP-1 and the percentage of lymphocytes ( $p = 0.006$ ). A negative relationship between the concentrations of IL-12 and IL-10 has been also observed ( $p = 0.028$ ). This research allows us to hypothesise that IL-10 may have a pathogenetic role in the evolution of asbestosis.

**Key words:** asbestosis, bronchoalveolar lavage, cytokines.

### Introduction

In the last decade experiments on guinea-pigs (22, 32, 16, 15) and observational studies of people exposed to asbestos (7, 11, 18, 33, 38) have helped to increase our knowledge about the immunopathogenetic mechanisms which regulate the cellular and humoral responses to inhaling asbestos fibres into the lungs.

In particular, it has been possible to define the role of inflammatory cytokines, cell growth factors, and oxidant radicals in the development of tissue damage and the consequent fibrogenesis (28). Most of the published studies have used bronchoalveolar lavage (BAL) as the technique for sampling cellular material and soluble fac-

tors from the alveolar environment (3, 10, 42, 42). BAL was a method introduced in the 1970's to study interstitial lung diseases (12) and although it is diagnostic for only some types of interstitial lung disease, it does play an important role in evaluating the activity of the disease and its prognosis as well as being a useful research tool. In the field of occupationally associated interstitial lung diseases, BAL may be indicated for the following purposes (11):

- to exclude other diffuse infiltrative diseases (sarcoidosis, alveolar haemorrhages, neoplasms, etc.) in patients occupationally exposed to respiratory hazard.
- to document that exposure to asbestos has indeed occurred in subjects without a strong occupational history,
- to study the local immune-mediated inflammatory processes (alveolitis).

The aim of this study was to determine the relationships between the level of asbestos bodies in BAL specimens with cell counts and cytokine parameters and how these parameters relate to pulmonary physiology, radiology and asbestos exposure.

## Materials and methods

We studied 25 subjects (24 males and 1 female) with a past occupational history of exposure to asbestos. The subjects were admitted to the Institute of Occupational Medicine of Pavia for diagnostic and medico-legal evaluations in the context of suspected asbestosis. The patients had worked in the following industrial sectors: ship construction (n=6); iron works (n=7); asbestos and cement works (n=7); other (n=5).

Their mean age ( $\pm$  s.e.) was  $57.4 \pm 1.84$  years (range 24-72 years) and the mean ( $\pm$  s.e.) exposure duration was  $24.5 \pm 2.28$  years (range 5-39).

An accurate occupational history was taken from all the subjects followed by careful clinical examination. The subjects were semiquantitatively classified into three risk groups, taking into account the probability, intensity and length of their exposure to asbestos:

- Mild exposure (n=9)
- Moderate exposure (n=8)
- Heavy exposure (n=8)

The patients' smoking habits were quantified on the basis of pack year and this information was used to divide the patients into 4 classes:

- 0 pack year (n=6)
- 1-20 pack year (n=5)
- 21-30 pack year (n=6)
- 31-50 pack year (n=8)

The patients also underwent the following instrumental investigations as part of their diagnostic investigations:

- **respiratory function tests:** vital capacity (VC), forced expiratory volume in one second (FEV1), Tiffeneau's index (FEV1/VC), residual volume (RV), and carbon monoxide diffusing capacity (DLCO) by single breath method, were measured with a spirometer (Master-screen FRC, Jaeger). The results of these lung function

tests were expressed as percentages of the minimum reference values given by the European Community for Coal and Steel (ECCS) tables (30).

Arterial blood-gas samples were studied using a blood gas analyzer (Synthesis 10, Instrumentation laboratory): the results were expressed as mmHg.

- **standard chest X-ray** (P-A and lateral projections) using digital type.

The chest X-rays were evaluated according to the International Labour Office (I.L.O.) classification by a single radiologist with expertise in this type of judgement.

- **high resolution computed tomography (HRCT) of the chest.**

The patients were divided on the basis of their radiological picture into 2 subclasses according to the I.L.O. classification:

**Class 0/0- 0/1:** parenchymal opacities absent or less profuse than in category 1 (n. 14)

**Class 1/0 and higher:** presence of increasingly profuse parenchymal opacities (n. 11)

- **bronchoscopy with bronchoalveolar lavage (BAL).**

The bronchoscopy was carried out using a Flex Pentax EBH bronchoscope or with an EB-1830T2 Pentax videobronchoscope.

The BAL was performed following the guidelines of the European Respiratory Society (21): three 50 ml aliquots of physiological saline (0.9% NaCl) were infused into the middle or lingular bronchus. Each of the three aliquots was reaspirated, collected into separate conical-bottomed test-tubes and then stored on ice. The second and third washings were used for the cytological and immunological studies. Once the BAL samples reached the laboratory they were filtered through sterile gauze (8, 21, 37).

The following evaluations of the BAL were carried out:

- 1) absolute and differential cell count: macrophage, lymphocyte, neutrophil and eosinophil granulocyte;
- 2) lymphocyte subpopulations: total lymphocytes (CD3), T helper lymphocytes (CD4), cytotoxic T lymphocytes (CD8), B lymphocytes (CD19), and activated T lymphocytes (HLADR);
- 3) concentration of the asbestos bodies in the BAL by means of optical microscopy,
- 4) supernatant levels of cytokines: interleukin 8 (IL-8), interleukin 10 (IL-10), interleukin 12 (IL-12) and monocyte chemotactic protein (MCP-1).

The total cell count was determined using a Burker chamber. Aliquots of 10 ml of sample were prepared and centrifuged at 1500 rpm for 15 minutes in order to separate the cells from the overlying supernatant. The supernatant was then concentrated to 50% of its volume by ultrafiltration and used for the cytokine assays. The pellet was resuspended in cold phosphate buffer saline (PBS) at pH 7.2, and then diluted or concentrated in order to obtain between 200,000 and 300,000 cells/ml; aliquots of 100  $\mu$ l of this suspension were used to set up the slides for the cytological examinations.

The slides were centrifuged by cytospin for 5 minutes at 500 rpm and then stained with an acid-base method (Diff-Quick) and examined by optical microscopy.

The same suspension of the initial cell pellet was used to determine the lymphocyte subclasses (CD3, CD4, CD8, CD19, HLADR) by flow cytometry (Ortho Cytoron) employing specific monoclonal antibodies (Ortho).

The following soluble components were assayed in the supernatant:

- **MCP-1:** the assay was carried out using an ELISA method with a pair of antibodies developed by R&D Systems (MN, USA). Human monoclonal anti-MCP-1 (clone 23007.1119) at a concentration of 1 µg/ml was used as the coating antibody whereas the detecting antibody was biotinylated human polyclonal anti-MCP-1 at a concentration of 50 ng/ml. The limit of sensitivity of the kit is 3.4 pg/ml of recombinant human MCP-1.
- **IL-8:** the assay was carried out using an ELISA method with a pair of antibodies developed by Immunokontakt (Frankfurt-am-Main). The coating antibody was human monoclonal anti-IL-8 (clone 3IL8-H10), used at a concentration of 1 µg/ml, and the detecting antibody was biotinylated human monoclonal anti-IL-8 (clone I8-S2) used at a concentration of 50 ng/ml. The limit of sensitivity of the kit is 1 pg/ml of recombinant human IL-8.
- **IL-10:** the assay was performed using an ELISA method with a pair of antibodies developed by Immunokontakt (Frankfurt-am-Main). The coating antibody was monoclonal anti-IL-10 (clone 9016.2), used at a concentration of 4 µg/ml, and the detecting antibody was biotinylated monoclonal anti-IL-10 (clone JES3-1248) at a concentration of 50 ng/ml. The limit of sensitivity of the kit is 1.6 pg/ml of recombinant human IL-10.
- **IL-12:** The assay was carried out using an ELISA method with a pair of antibodies developed by Endogen (Woburn USA). Monoclonal anti-IL-12 (clone 20C2) at a concentration of 4 µg/ml was used as the coating antibody and biotinylated monoclonal anti-IL-12 (clone C8.6) at a concentration of 1 µg/ml as the detecting antibody. The limit of sensitivity of the kit is 3 pg/ml of recombinant human IL-12.

The reference values for the cell components and soluble factors in the BAL were those obtained from an internal reference group tested in the clinical immunology laboratories of the Fondazione Salvatore Maugeri and the Institute of Respiratory Diseases of the University of Pavia.

The mineralogical studies and identification of any asbestos bodies were carried out by optical microscopy (14) of specially prepared membranes as well as directly on the cytospin. To this aim, 10 ml of previously not filtered BAL were centrifuged at 1500 rpm for 10 minutes and the sediment obtained resuspended in 10 ml of sodium hypochlorite 10% (sodium hydrate and hypochlorite solution, Carlo Erba) and then incubated for about 24 hours at a constant temperature of 40°C in order that complete cell digestion of all organic components took place. The solution was then filtered through a cellulose nitrate membrane filter with a mean porosity of 0.8 µm and a diameter of 25 mm, using Luer Lock syringes with a Millipore adapter. The membranes prepared in this way were diaphanised with hot acetone vapours, mounted on slides, added with

two drops of glycerol triacetate (triacetine) and covered with a cover slip. After a two hours delay they were examined under the optical microscope.

For the scan electron microscopy (SEM) examination the membrane filter, prepared with the previously described method, were diaphanised with hot acetone vapours on aluminium support (stub) and subsequently submitted to a vacuum deposition on the surface of a thin carbon layer (graphite), to obtain a good electrical conductivity. Finally the membrane was observed by the electron microscope (SEM Leica, Mod. STEREOSCAN 430), equipped with a X-ray analysis of elements (energy dispersion). The analysis was performed with a 2000x magnification, examining 1000 fields (25).

The examined subjects have been classified into two groups according to the presence or absence of asbestosis bodies (AB) in the BAL:

- AB absent (n=12)
- AB present (n= 13)

The latter has been further subdivided into two subgroup according to the concentration of AB in the BAL:

- ≤10 AB / ml (n= 7)
- > 10 AB / ml (n= 6)

#### Statistical analysis

The chi-squared test ( $\chi^2$ ) (Mantel-Haenzel test) was used to evaluate the associations between the evaluated risk factors (degree of exposure, asbestos bodies in the BAL), the severity of the radiological picture and smoking habits.

The comparisons of the variables between the groups, identified according to their risk factors, were done by student T test, analysis of the variance and the post-hoc test for those variables which emerged as being statistically significant. The value of each variable has been expressed as mean±standard error. Regression analysis was used to evaluate the association between cytological parameters, cytokine assays and respiratory function.

The level considered to be statistically significant in all tests was 0.05.

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#### Results

Of the group of patients examined, 11 did not have any respiratory symptoms, while 14 had symptoms (12 dyspnoea, 2 cough). 17 subjects had normal, 4 restrictive syndrome and 4 obstructive patterns spirometry.

The table I shows the descriptive statistics for the cellular and soluble components of the BAL and the respiratory function data.

Comparing the non-smokers and smokers, the former had higher levels of pO<sub>2</sub> (p<0.05), but no significant differences were found in lung volumes, cell components or soluble factors in the BAL.

Smoking habits were uniformly distributed between the subclasses of subjects defined according to degree of exposure and I.L.O. profusion asbestosis bodies in the BAL ( $\chi^2$  test: not significant). Thus smoking was not considered in this sample population as a confounding factor in the comparisons and the subsequently investigated relationships.

**Table 1. Descriptive statistics of the BAL and respiratory function parameters in the studied subjects**

	MEAN ± STANDARD ERROR	MEDIAN	RANGE MIN/MAX	REFERENCE VALUES
Cells/ml x 10 <sup>4</sup>	33.54 ± 3.94	26	6.0/81.0	< 20 x 10 <sup>4</sup>
Macrophages %	88.34 ± 2.40	94.7	58.0/99.0	>85%
Lymphocytes %	7.63 ± 1.59	3.8	.30/29.0	<13%
Neutrophils %	3.36 ± 1.59	.8	0.0/36.8	<3%
Eosinophils %	0.49 ± 0.11	.2	0.0/1.6	<0.5%
CD-3 %	58.26 ± 4.98	65	5.5/89.0	63-83%
CD-4 %	33.82 ± 3.59	33	1.8/76.0	40-60%
CD-8 %	23.61 ± 2.56	24	2.5/45.0	20-40%
CD-4/CD8 %	1.73 ± 0.22	1.3	.44/.44	1.2 - 2.5
CD-19 %	0.88 ± 0.78	.7	0.0/3.0	0-4%
HLA-DR %	15.95 ± 2.39	12.8	1.3/36	< 5%
MCP-1 pg/ml	26.07 ± 7.29	13.99	0/108.9	0 - 21.17
IL-8 pg/ml	29.93 ± 9.52	10.88	0.0/178.9	0 - 17.1
IL-10 pg/ml	27.17 ± 8.12	17.68	0.0/149.2	0 - 24.5
IL-12 pg/ml	13.24 ± 1.81	12.92	1.99/30.22	0 - 10.45
VC (L)	3.86 ± 0.15	3.90	2.40/5.50	-
VC% pred.	119.2 ± 4.49	123.0	73.0/176.0	-
FEV1 (L)	2.87 ± .137	2.81	1.8/4.7	-
FEV1% pred.	126.52 ± 5.06	121.0	85.0/194.0	-
FEV1/VC%	74.16 ± 1.39	75	58.0/85.0	-
DLCO mlCOxmmHg/min	29.5 ± 1.16	27.25	21.0/42.0	-
DLCO% pred.	113.39 ± 3.74	116	82.3/147.0	-
PO2 mmHg	77.78 ± 1.84	76	65.0/98.0	-

### Degree of exposure

The correlations between the cytological parameters of the BAL (total cell count, percentage of macrophages, lymphocytes, granulocytic neutrophils and eosinophils) and the degree of exposure were evaluated. A statistically significant ( $p < 0.05$ ) higher percentage of lymphocytes ( $13.07 \pm 2.31\%$ ) was found in the BAL from subjects with moderate exposure, in comparison with the mild ( $2.53 \pm 2.18\%$ ) and heavy ( $7.07 \pm 2.31\%$ ) exposure groups. No statistically significant correlations emerged between the concentrations of MCP-1, IL-8, IL-10 and IL-12 in the bronchoalveolar lavage and either the degree of exposure. Moreover we didn't find any significant difference comparing the degree of exposure to both the respiratory functional parameters and the radiological picture.

### BAL Mineralogical study

We investigated whether there was any relationship between the concentration of asbestos bodies (AB) in the bronchoalveolar lavage fluid and degree of exposure, cytological and humoral parameters of the BAL, the radiological picture and respiratory function.

The levels of AB in the BAL was not related to the degree of occupational exposure to asbestos, the radiological

picture nor to respiratory function parameters. There were no statistically significant correlations between the concentration of AB and cytological findings in the BAL, while a significant increase of IL-10 concentration has been found in subjects without AB in the BAL fluid (AB absent:  $42.73 \pm 13.44$  pg,  $p < 0.05$ ) compared to the others (AB present:  $10.05 \pm 5.0$  pg); the latter showed an increase of IL-8 of border line significance (Fig. 1a). Finally a further evaluation of cellular and soluble components of the BAL, according to the concentration of asbestos fibres ( $\leq 10$  AB/ml vs  $< 10$  AB/ml), did not show significant differences.

Ultramicroscopy and analysis of the asbestos bodies in the BAL was carried out by X-ray scanning electron microscopy in one patient. This confirmed the presence of asbestos fibres, identified as amosite fibres (Photo 1a, b).

### Radiological picture

Comparing the cytological parameters of the BAL with the radiological picture, a statistically higher percentage of lymphocytes was found in the group with the radiological I.L.O classification 0/0-0/1 ( $10.57 \pm 2.46\%$ ,  $p < 0.02$ ), compared to in those with pleuro-parenchymal lesions ( $3.27 \pm 0.89\%$ ) (Fig 1b). There was a more marked alveolar neutrophilia in the subjects with overt pulmonary fi-

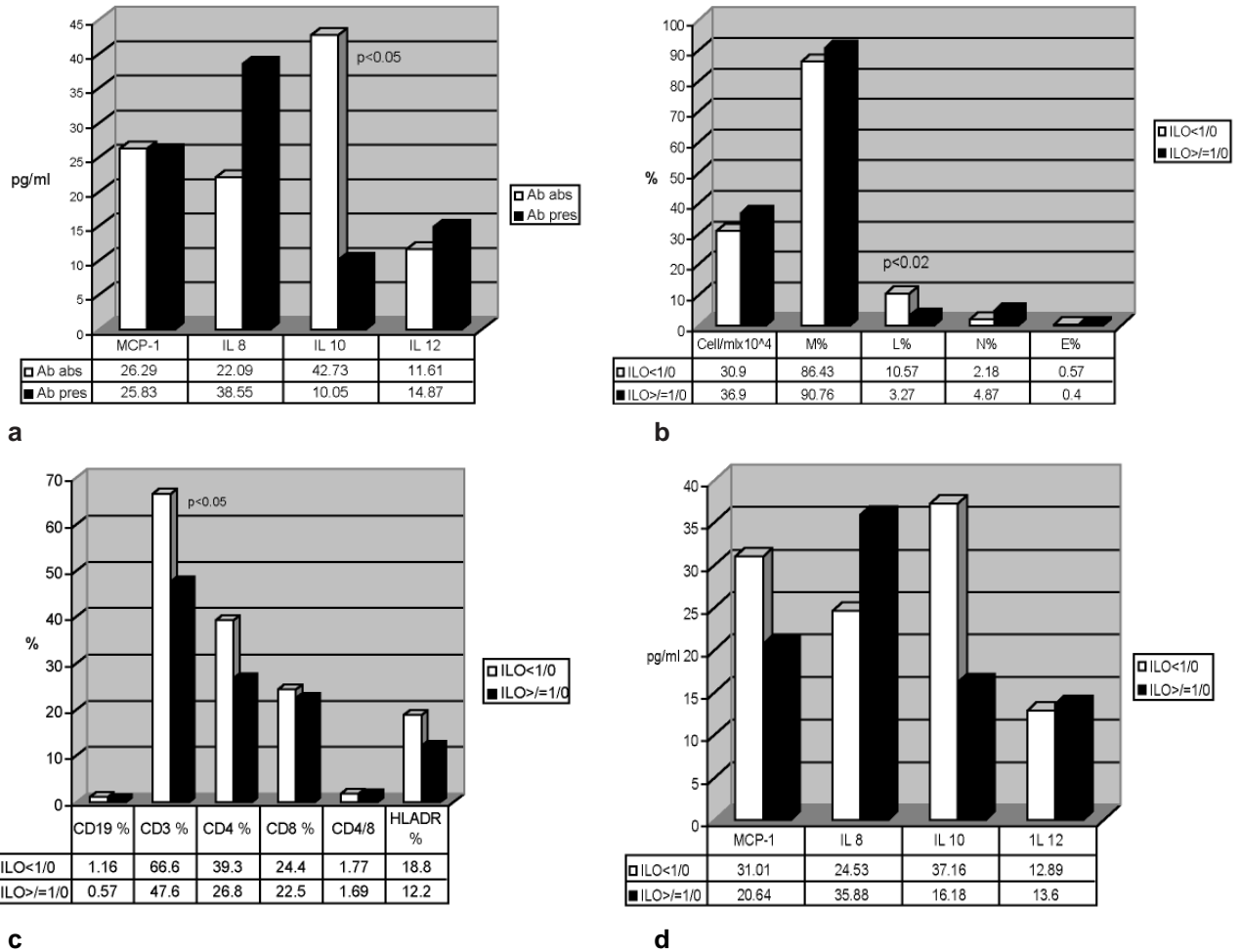


Figure 1. Comparison of cellular and humoral components of BAL according to the AB concentration in BAL and to the radiological picture.

The value of each variable is expressed as mean in the tables.

- a) Concentration of cytokines according to the presence of asbestos bodies (AB) in the BAL (white bar=AB present; black bar=AB absent)
- b) Cellular profile of BAL according to the radiological picture (M= macrophages; L=lymphocytes; N=neutrophils; E=eosinophils; white bar=I.L.O.<1/0; black bar=I.L.O. ≥1/0)
- c) Subsets lymphocyte in BAL according to the radiological picture (white bar=I.L.O.<1/0; black bar=I.L.O. ≥1/0)
- d) Concentration of cytokines in the BAL according to the radiological picture (white bar=I.L.O.<1/0; black bar=I.L.O. ≥1/0)

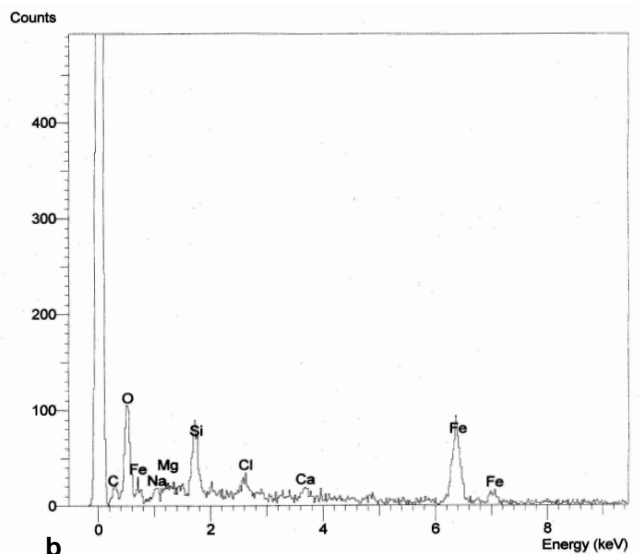
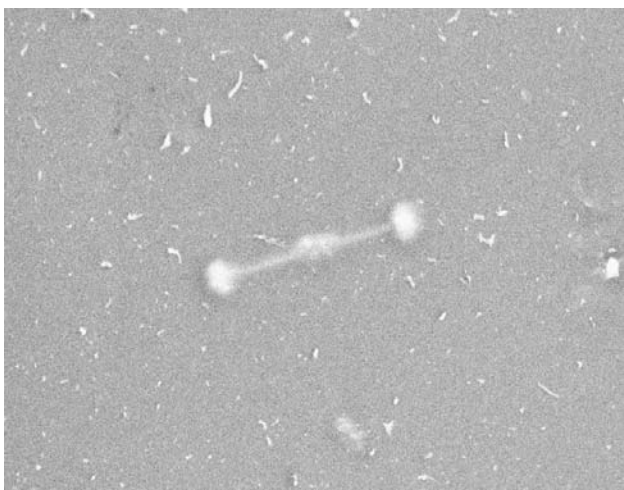


Photo 1. Asbestos fiber (a), detected in the BAL from a patient affected with asbestosis. S.E.M. microanalysis trace (b) of the characteristic elements of the amosite fiber (a)

brosis and in one patient with typical bilateral crackles at the lung lower lobes but without radiological signs of interstitial disease. Evaluation of the lymphocyte subclasses showed a significant increase in total T lymphocytes (CD3) ( $66.6 \pm 6.88\%$  -  $p < 0.05$ ) in comparison to in the group with a  $\geq 1/0$  picture ( $47.65 \pm 4.02\%$ ). Moreover, despite an increase of T helper cells (CD4) ( $39.34 \pm 5.24\%$ ) of borderline significance ( $p = 0.05$ ) in the group of subjects with a 0/0-0/1 radiological picture, any significant difference in the CD4/CD8 ratio has been observed (Fig. 1c).

Comparing the BAL concentration of the cytokines according to the radiological picture, a trend for higher levels of IL-10 and MCP-1 in subjects with a 0/0-0/1 radiological picture (Fig 1d) was found, whereas IL-8 tended to be higher in the group with overt pulmonary fibrosis (I.L.O.  $\geq 1/0$ ).

Moreover the subjects with an advanced radiological picture (I.L.O.  $\geq 1/0$ ) had a significantly lower VC%pred. ( $108.1 \pm 5.47\%$  -  $p < 0.05$ ) and FEV1%pred. ( $113.5 \pm 5.12\%$  -  $p < 0.02$ ) than the subjects showing fewer radiological changes ( $127.9 \pm 5.94\%$  and  $136.7 \pm 7.11\%$ , respectively).

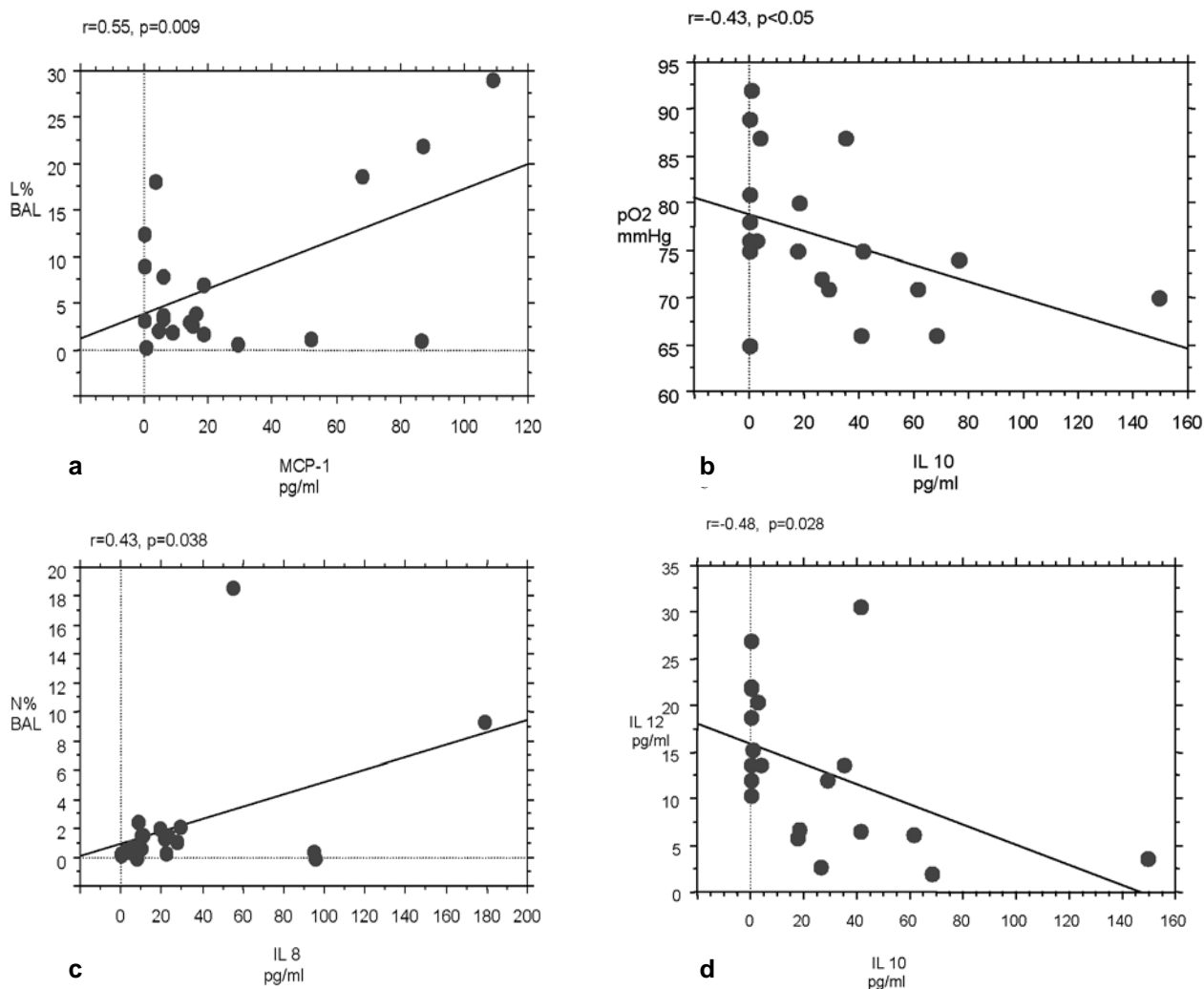
### Regression analysis

There was a statistically significant positive correlation between the concentration of MCP-1 and the percentage of lymphocytes in the BAL ( $r = 0.55$ ,  $p = 0.006$ ) (Fig. 2a), while the concentration of IL-10 was inversely correlated with pO<sub>2</sub> ( $r = -0.43$ ,  $p = 0.048$ ) (Fig. 2b). IL-8 was correlated ( $r = 0.43$ ,  $p = 0.038$ ) with the percentage of granulocytic neutrophils in the BAL (Fig. 2c). The concentration of IL-12, on the other hand, was inversely correlated with the concentration of IL-10 ( $r = -0.48$ ,  $p = 0.028$ ) (Fig. 2d).

The comparison of the BAL cytology and respiratory function showed a negative and statistically significant correlation between the percentage of granulocytic neutrophils and both vital capacity VC(%pred.) ( $r = -0.41$ ,  $p = 0.03$ ) and partial pressure of oxygen (pO<sub>2</sub>) ( $r = -0.3$ ,  $p = 0.05$ ).

### Discussion

Numerous studies were carried out, particularly in the 1980s, with the aim of demonstrating early changes in the



**Figure 2. a) Relationship between the concentration of MCP-1 (x axis) and Lymphocytes % (L) (y axis) in the BAL**  
**b) Relationship between the concentration of IL-10 in the BAL (x axis) and pO<sub>2</sub> (y axis)**  
**c) Relationship between the concentration of IL-8 (x axis) and Neutrophils % (L) (y axis) in the BAL**  
**d) Relationship between the concentration of IL-10 (x axis) and IL-12 (y axis). in the BAL.**

bronchoalveolar lavage of subjects exposed to asbestos and of identifying more sensitive markers, either in the cell component or the soluble part, of lung damage than radiological findings or respiratory function tests.

Law no. 257/92 has largely prohibited asbestos processing in Italy and our investigation have shown bronchoalveolar changes in patients with past occupational exposure to asbestos. Moreover, we evaluated the alveolar concentration of some cytokines (such as IL-10 and IL-12), not yet documented in the literature with regard to its relationship to exposure to asbestos.

Our study showed a homogeneous distribution of smokers within the various classes of exposure and I.L.O. radiological pattern; for this reason smoking history was not considered a confounding factor. Other authors have observed that in the BAL of subjects exposed to asbestos, cigarette smoking contributes more to the increase in macrophages and eosinophils, while the percentages of lymphocytes and neutrophils were not significantly affected (35).

The immunocytological evaluations of the bronchoalveolar lavage showed a significantly higher percentage of lymphocytes in the group of subjects with a moderate degree of exposure and a radiological picture characterised predominantly by pleural plaques, compared to subjects in the other groups of exposure and subjects with pulmonary fibrosis.

Notwithstanding the limitations due to the small population sample, these results were in agreement with those obtained elsewhere (10, 11); in fact, the most common finding in the BAL of subjects with documented exposure to asbestos but without signs of pulmonary fibrosis was a lymphocytic alveolitis. An increased alveolar lymphocytosis also seemed to be associated with pleural plaques and a more stable radiological picture over time (39, 41). This lymphocytic alveolitis was characterised by an expansion of the T helper subpopulation with an increase in the CD4/CD8 ratio and in surface markers of cellular activation (34, 38, 41). A study by Rom *et al.* (33) documented a spontaneous release of IFN from the BAL cells of subjects exposed to asbestos, suggesting a polarized Th1 response occurring in early stage of the disease. Nevertheless, the significance of this isolated increase in alveolar lymphocytes was not completely clear in the case of asbestosis.

A study by Corsini *et al.* showed that genetically immunodeficient rats (BALB/c/nu/nu) exposed to asbestos had a higher cell count (predominantly neutrophils) in the BAL, and a higher concentration of leukotriene B4 (LTB4), prostaglandin E2 (PGE2), fibronectin and hydroxyproline than rats of the same strain reconstituted with syngeneic lymphocytes, suggesting that T lymphocytes have a protective role in the pulmonary inflammation and collagen deposition caused by the inhalation of asbestos (9). Confirmation of this hypothesis comes from a study by Gellert who identified a subgroup of subjects with T lymphocytic alveolitis and a better lung function profile from among an overall group of workers with asbestosis (18).

In the study by Al Jarad and colleagues (1993) the radiological progression and annual decrease in lung function (in particular lung diffusion) was inversely related to the percentage of lymphocytes in the bronchoalveolar fluid (3).

In subjects with advanced radiological disease, characterised by signs of pulmonary fibrosis, there was a trend towards a higher cell count/ml and a higher percentage of granulocytic neutrophils.

This last result fits in with the natural history of fibrotic processes of the lung and is not specific to asbestosis. The presence of polymorphonuclear cells infiltrating the lung parenchyma is normally associated with fibrotic disorders such as idiopathic pulmonary fibrosis and collagen diseases. The neutrophils, attracted to sites of inflammation by cytokines released by macrophages and lymphocytes (IL-8, LTB4) (34), play an important role in the phase of parenchymal damage by releasing proteolytic enzymes (such as elastases and collagenases), myeloperoxidases and free radicals. These cells do not, however, have much influence of the phases of lung repair and remodelling because they neither release fibroblast growth factors nor stimulate the production of collagen fibres (36).

Observations of cohorts of workers have shown, albeit not unequivocally, an increase in neutrophils in the BALF of subjects with asbestosis compared to in those only exposed to this mineral, highlighting the correlation between neutrophil alveolitis and greater radiological changes and more pronounced deterioration of respiratory function in longitudinal studies (13, 18, 42). A study by Robinson in 1986 (31), despite showing a correlation between the percentage of neutrophils in the BAL and the duration of exposure to asbestos, did not demonstrate a significant difference in the severity of the alveolitis among subjects with radiological and functional changes associated with asbestosis and subjects only exposed to the mineral or with crepitant rales at chest auscultation. We too found a negative correlation between the percentage of granulocytic neutrophils and some lung function parameters (VC and pO<sub>2</sub>), but we also documented a marked alveolar neutrophilia in one subject without fibrotic type radiological changes and with lung function tests within the normal range.

The more recent studies on interstitial lung diseases attribute an important pathogenetic role to the equilibrium between the T helper1 (Th1) and T helper2 (Th2) lymphocyte subclasses; the former is implicated particularly in mechanisms of delayed immune responses and produces interleukin 2 (IL-2), 12 (IL-12) and interferon  $\gamma$  (INF $\gamma$ ), while the latter has a role in regulating the immediate immune response, particularly IgE-mediated responses, and produces interleukin 4 (IL-4), 5 (IL-5), 6 (IL-6) and 10 (IL-10) (1).

It is thought that an imbalance in this ratio, with an expansion of the Th2 lymphocytes, enhances the fibrogenetic response mediated by Th2-associated cytokines (2).

The evaluation of the cytokines in the BAL yielded interesting information.

Interleukin-10, a cytokine synthesised predominantly by Th2 lymphocytes, but also by monocytes, macrophages and B lymphocytes, has an inhibitory effect of various immune-mediated mechanisms such as proliferation of T lymphocytes, production of Th1 cytokines (IL-2 and INF $\gamma$ ), antigen presentation and cytotoxicity of natural killer cells (27). Its concentration in the BAL tended to be higher in subjects showing alveolar lymphocytosis and ab-

sence of asbestos bodies in the BAL with no evidence of pulmonary fibrosis on the chest x ray.

The interpretation of this result is not immediately obvious. In fact, on the one hand the increase of IL-10 in patients having a I.L.O. radiological classification <1/0 and absence of Ab in the BAL, could represent a good prognostic factor, as expression of a greater secretion by macrophages and alveolar lymphocytes aimed at inhibiting the production of inflammatory cytokines, particularly TNF  $\alpha$  and INF $\gamma$ , which are increased in the BAL of patients with asbestosis (32). On the other hand, the documented negative correlation with the pO<sub>2</sub> suggests an important role in the genesis of parenchymal damage, in consequence of the expansion of the Th2 subclass associated with the fibrotic process and the inhibition of antifibrotic Th1 cytokines.

Although we are not aware of other studies concerning this cytokine in asbestosis, there are two studies on the lung response of rats to inhalation of silica particles. These studies show that the increased synthesis of IL-10 induced by the silica limits the extent of the inflammatory response and the production of TNF $\alpha$ , but stimulated the fibrotic response (19, 20). Other authors, using bronchoalveolar lavage to study subjects with idiopathic pulmonary fibrosis and bronchiolitis obliterans with organising pneumonitis (BOOP), have found, in fibrotic patients, the same or even lower alveolar concentrations of IL-10 as those in BOOP and in control subjects (24, 26), despite there being increased expression of m-RNA for IL-10. The authors offer various explanations for this apparent contradiction: increased breakdown by proteolytic enzymes, uptake by cell surface receptors, increased expression of IL-10 on the cell membrane of macrophages (24).

In vitro and in vivo studies in man have shown a substantial increase in IL-8 and an important role for this cytokine in the genesis of the neutrophil alveolitis which characterises asbestosis (6, 34). This chemokine is produced by numerous cells, including macrophages, monocytes, activated CD4 and CD8 T lymphocytes, epithelial cells, and keratinocytes. It has a chemotactic effect on neutrophils and is particularly involved in the early stage of inflammation. Furthermore, some experimental studies have shown a direct association between IL-8 release and the development of lung fibrosis. In fact IL-8, synthesised by macrophages, plays an important role in the recruitment of neutrophils during idiopathic pulmonary fibrosis. Furthermore, it could indirectly enhance chemotaxis of T cells and production of cytokines, inducing degranulation of neutrophils (40). In our study the concentration of this cytokine was correlated ( $p=0.03$ ) with the percentage of neutrophils in the BAL, which showed a negative relationship with vital capacity and pO<sub>2</sub>.

The cytokine MCP-1 seems to have a role in the recruitment of T lymphocytes, but we are unaware of studies concerning this cytokine in asbestosis. In our study its concentration correlated with the percentage of lymphocytes and tends to be higher in patients with less severe radiological signs.

Interleukin 12 is a cytokine produced by dendritic cells. It stimulates the production of INF $\gamma$  by T lymphocytes

and natural killer cells, stimulates the proliferation of lymphocytes in the blood and induces the differentiation of Th1 subpopulations of lymphocytes which produce IL-2 and INF $\gamma$ .

Recent studies have shown that IL-12 has an important role in the development of granulomatous diseases but there have been no studies on its relevance in exposure to asbestos fibres. However, one experimental study of silica exposure in rats demonstrated that this cytokine has an antifibrotic effect on the lung due to inhibition of the Th2 subpopulation of lymphocytes (19) and this is in agreement with the inverse correlation between IL-10 and IL-12 documented in our study.

In conclusion, this study has confirmed that bronchoalveolar lavage is useful in diagnosing lung diseases associated with the inhalation of asbestos fibres. It allowed us to document exposure to asbestos in particular cases in which the occupational history was not clear. Nevertheless, careful history taking remains the irreplaceable cornerstone to the approach to occupational lung diseases and interstitial lung diseases in general (4).

This study has also shown an inflammatory phase in early stages of asbestosis, characterized by a lymphocytic alveolitis and related to the concentration of MCP-1 in the bronchoalveolar lavage specimens. In more advanced stages, an alveolar neutrophilia, related to the BAL levels of IL-8, seemed to be present. The role of IL-10 in the regulation of inflammatory and fibrotic events following exposure to asbestos remains unclear, but our observations, in agreement with earlier works (19, 20, 24, 29), suggest both an antiinflammatory and a profibrotic action. Nevertheless, because of the small sample size, these results need to be confirmed in a larger group of subjects.

## References

- 1) Agostini C, Chilosi M, Zambello R, Trentin L, Semenzato G. Pulmonary immune cells in health and disease; lymphocytes. *Eur Respir J* 1993; 6: 1378-1401.
- 2) Agostini C, Siviero MD, Semenzato GP. Immune effector cells in idiopathic pulmonary fibrosis. *Current Opinion in Pulmonary Medicine* 1997; 3: 348-355.
- 3) Al Jarad N, Gellert AR, Rudd RM. Bronchoalveolar lavage and <sup>99m</sup>Tc-DTPA clearance as prognostic factors in asbestos workers with and without asbestosis. *Respir Med* 1993; 87: 365-374
- 4) American Thoracic Society. Idiopathic pulmonary fibrosis; diagnosis and treatment. *Am J Respir Crit Care Med* 2000; 161: 646-664.
- 5) Biscaldi G, Fonte R, Paita L, Vittadini G, Caprotti M. La tomografia computerizzata ad alta risoluzione nella diagnosi di asbestosi. *G Ital Med Lav Erg* 1999; 21, 4: 271-277.
- 6) Broser M, Zhang Y, Aston C, Harkin T, Rom WN. Elevated interleukin-8 in the alveolitis of individuals with asbestos exposure. *Int Arch Occup Environ Health* 1996; 68: 109-114.
- 7) Brown SK. Asbestos exposure during renovation and demolition of asbestos-cement clad buildings. *Am Ind Hyg Assoc J* 1987; 48: 478-486.
- 8) Capelli A, Balbi B, Brunetti G, Confalonieri M, Lusuardi M, Marchesani F, Pesci A, Poletti V, Spanevello. Standard tecnico-operativi del lavaggio broncoalveolare. *Rassegna di patologia dell'apparato respiratorio* 1998; 13: 160-164.
- 9) Corsini E, Luster MI, Mahler J, Craig WA, Blarka ME, Rosenthal GJ. A protective role for T-lymphocytes in asbestos-induced pulmonary inflammation and collagen deposition. *Am J Respir Cell Mol Biol* 1994; 11: 531-539.

- 10) Costabel U, Brooks KJ, Huck E, Gurman J, Matthys M. Lung and blood lymphocyte subsets in asbestosis and in mixed dust pneumoconiosis. *Chest* 1987; 91: 110-112.
- 11) Costabel U, Donner CF, Haslam PL, Rizzato G, Teschler H, Velluti G, Wallaert B. Occupational lung diseases due to inhalation of organic dust. *Eur Resp J* 1990; 3: 946-949.
- 12) Costabel U. Atlas of bronchoalveolar lavage, Chapman and Hall Medical 1998.
- 13) Cullen M, Merrill W. Association between neutrophil concentration in bronchoalveolar lavage fluid and recent losses in diffusing capacity in men formerly exposed to asbestos. *Chest* 1992; 102: 682-687.
- 14) De Vuyst P, Dumortier P, Moulin E, Yourossowsky, Yernault JC. Diagnostic value of asbestos bodies in bronchoalveolar lavage fluid. *Am Rev Respir Dis* 1987; 136: 1219-1224.
- 15) Driscoll KE, Maurer JK, Higgins J, Poynter J. Alveolar macrophage cytokine and growth factor production in rat model of crocidolite-induced pulmonary inflammation and fibrosis. *J Toxicol Environ Health* 1995; 46(2): 155-169.
- 16) Dubois MC, Bissonnette E, Rola-Pleszczynski M. Asbestos fiber and silica particles stimulate rat alveolar macrophages to release tumor necrosis factor. *Am Rev Respir Dis* 1989; 139: 1257-1264.
- 17) Gellert AR, Macey MG, Uthayakumar S, Newland AC, Rudo RM. Lymphocyte subpopulations in bronchoalveolar lavage fluid in asbestos workers. *Am Rev Respir Dis* 1985; 132: 824-828.
- 18) Gellert AR, Langford JA, Winter RJD, Uthayakumar S, Sinha G, Rudd RM. Asbestosis; assessment by bronchoalveolar-lavage and measurement of pulmonary epithelial permeability. *Thorax* 1985; 40: 508-514.
- 19) Huaux F, Lardot C, Arras M, Delos M, Many MC, Coutelier JP, Buchet JP, Renauld JC, Lison D. Lung fibrosis induced by silica particles in NMRI mice is associated with an upregulation of the p40 subunit of interleukin-12 and Th-2 manifestations. *Am J Respir Cell Mol Biol* 1999; 20: 572-581.
- 20) Huaux F, Louahed J, Hudspith B, Meredith C, Delos M, Renauld JC, Lison D. Role of interleukin-10 in the lung response to silica in mice. *Am J Respir Cell Biol* 1998; 18: 51-59.
- 21) Kleck H, Pohl W. Technical recommendations and guidelines for bronchoalveolar lavage (BAL). *Env Respir J* 1989; 2: 561-585.
- 22) Lemaire I, Beaudoin H, Dubois C. Cytokine regulation of lung fibroblast proliferation. Pulmonary and systemic changes in asbestos-induced pulmonary fibrosis. *Am Rev Respir Dis* 1986; 134(4): 653-658.
- 23) Lemaire I. Characterization of the bronchoalveolar cellular response in experimental asbestosis. *Am Rev Respir Dis* 1985; 131: 144-149.
- 24) Martinez JA, King TE Jr, Brown K, Jennings CA, Borish L, Mortenson RL, Khan TZ, Bost Riches DW. Increased expression of the interleukin-10 gene by alveolar macrophages in interstitial lung disease. *Am J Physiol* 1997; 273: 676-683.
- 25) Massola A. Analisi al microscopio elettronico a scansione (SEM) delle fibre aerodisperse and depositate su membrana micropori. 1997. L'asbestos; dall'ambiente di lavoro all'ambiente di vita. Nuovi indicatori per futuri effetti. 217-224.
- 26) Forlani S, Rata L, Bulgheroni A, Cascina A, Paschetto E, Cervio G, Luinetti O, Fietta AM, Meloni F. Cytokine profile of broncho-alveolar lavage in BOOP and UIP. Sarcoidosis Vsc. *Diffuse Lung Dis* 2002; 19: 47-53.
- 27) Moore KW, O'Garra A, De Wall Malefyt R; Interleukine 10. *Ann Rev Immunol* 1993; 11: 165-190
- 28) Mossman BT, Churg A. Mechanisms in the pathogenesis of asbestosis and silicosis. *Am J Respir Crit Care Med* 1998; 157: 1666-1680.
- 29) Nemery B, Bast A, Behr J, Borm PJA, Bourke SJ, Camus Ph, De Vuyst P, Jansen HM, Kinnula VL, Lison D, Pelkonen O, Saltini C. Interstitial lung disease induced by exogenous agents: factors governing susceptibility. *Eur Respir J* 2001; 32: 30s-42s.
- 30) Quanjen H, Tammeling GJ, Cotes JE, Pedersen OF, Peslin R, Yernault JC; Lung Volumes and forced ventilatory flows. *Eur Respir J* 1993; 6: 15-40
- 31) Robinson BW, Rose AH, James A, Whitaker D, Musk AW; Alveolitis of pulmonary asbestosis. *Chest* 1986; 90(3): 396-402.
- 32) Robinson BW, Rose AH, Hayes A, Musk AW. Increased pulmonary gamma interferon production in asbestosis. *Am Rev Resp Dis* 1988; 138: 278-283.
- 33) Rom WN, Travis WD. Lymphocyte-macrophage alveolitis in non-smoking individuals occupationally exposed to asbestos. *Chest* 1992; 101: 779-786.
- 34) Rosenthal GJ, Germolec DR, Blazka ME, Corsini E, Simeonova P, Pollock P, Kong LY, Kwon J, Luster MI. Asbestos stimulates IL-8 production from human lung epithelial cells. *J Immunol* 1994; 153: 3237-3244.
- 35) Schwartz DA, Galvin JR, Merchant RK, Dayton CS, Burmeister LF, Merchant JA, Hunninghake JW. Influence of cigarette smoking on bronchoalveolar lavage cellularity in asbestos-induced lung disease. *Am Rev Respir Dis* 1992; 145: 400-405.
- 36) Sibille Y, Marchandise FX. Pulmonary cells in health and disease; polymorphonuclear neutrophils. *Env Respir S* 1993; 6: 1529-1543.
- 37) Spada EL, Lusuardi M, Capelli A, Zaccaria S, Donner CF. Modalità d'esecuzione del bronchoalveolar lavage (BAL); pretrattamento dei fluidi recuperati. *Rassegna di Patologia dell'Apparato Respiratorio* 1988; s; 21-24.
- 38) Sprince NL, Oliver LC, McLoud TC, Eisen EA, Christiani DC, Ginns LC. Asbestos exposure and asbestos-related pleural and parenchymal disease. *Am Rev Respir Dis* 1991; 143: 822-823.
- 39) Sprince NL, Oliver LC, McLoud TC, Morris TA, Tilles DS, Eisen EA, Ginns LC. T-lymphocyte subset in bronchoalveolar lavage and peripheral blood in asbestos markers; correlations with exposure and pleural plaques. *Chest* 1987; 91: 309.
- 40) Taub DD, Anver M, Oppenheim JJ. T lymphocyte recruitment by interleukin 8 (IL-8); IL8-induced degranulation of neutrophils releases potent chemoattractants for human T lymphocytes both in vitro and in vivo. *J Clin Invest* 1996; 97: 1931-1941.
- 41) Wallace JM, Oishi JS, Barbers RG, Batr P, Aberle DR. Bronchoalveolar lavage cell and lymphocyte phenotype profiles in healthy asbestos-exposed shipyard workers. *Am Rev Respir Dis* 1989; 139 (1): 33-38.
- 42) Xaubet A, Rodriguez-Roisin R, Bombi JA, Marin, Roca J, August-Vidal A. Correlation of bronchoalveolar lavage and clinical and functional findings in asbestosis. *Am Rev Respir Dis* 1986; 133: 848-854.

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