In situ analysis by SEM-EDX spectroscopy of 10 sarcoidosis cases from MINASARC study

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ERS INTERNATIONAL CONGRESS 2017, MILAN Italy, 9-13 september

I. Introduction

MINASARC is a case-control study (20 cases and 20 healthy volunteers) designed to assess the inorganic “exposome” by a specific questionnaire concomitantly with the mineralogical analysis (MA) of Broncho-Alveolar Lavage (BAL) fluid by transmission electron microscopy (TEM). One of the secondary objectives of this study is to compare those results with an *in situ* analysis of some paraffin embedded biopsies by scanning electron microscopy (SEM). We report data from 10 sarcoidosis patients corresponding to the 10 biopsies available for analysis.

II. Material and methods

A. Patients (Table 1)

Criteria for inclusion and exclusion of Sarcoidosis patients

Inclusion: suspected sarcoidosis stages 1-4; age 20-50; having signed an informed consent; being submitted to an endoscopic examination with BAL; without probable causal factor already identified during the usual patient interview; accepting to complete a professional and environmental questionnaire once sarcoidosis is confirmed.

Exclusion: refusing the terms of the informed consent; refusing to complete a professional and environmental questionnaire; non-completion or failure of BAL.

Table 1 : Demographic and clinical characteristics of the sarcoidosis patients. PN: patient number; Smo Sta: smoking status (0:< 5 PY; 1: 5 to 10 PY; 2: > 10PY); Stage: radiologic stage of the disease; EBUS: endobronchial ultrasound; TBNA: trans-bronchial needle aspiration; BB: bronchial biopsy; SAGB: salivary gland biopsy; LB: liver biopsy; SB: skin biopsy.

PN	Age	Sex	Country of birth	Smo Sta	Current occupation	Stage	Sampling type
1	40	M	France	0	Public relation worker	2	EBUS/TBNA
2	40	M	United States	0	Unemployed	1	EBUS/TBNA
3	29	F	France	0	Public service worker	1	EBUS/TBNA
4	29	F	France	0	Manicurist	1	EBUS/TBNA
5	29	M	France	0	Medical doctor	2	EBUS/TBNA
6	26	M	France	1	Stonemason	2	BB
7	39	M	France	0	Baker	2	BB
8	38	M	Cameroon	0	Tailer	2	BB, SAGB and LB
9	48	M	Portugal	1	Stonemason	1	BB and SAGB
10	39	M	Angola	0	Fire safety worker	4	BB and SB

B. MA by Electron Microscopy

1. Analysis of BAL fluid in TEM

The BAL’s MA was performed by transmission electron microscopy (TEM, Jeol 1400 EX, operated at 120 kV) on a device equipped with a CCD camera (Gatan Orius 600) and an X-ray emission detector (Jeol JED-2300). EDX spectra were acquired of a tile without the filter and a tile with a blank filter, to verify that there was no contamination. A global spectrum was then acquired of virtually the whole tile at ×5000 magnification, to assess the homogeneity of the grid. Finally, 100 contiguous particles were analyzed by EDX, starting in the central tile. The corresponding spectra were saved for further analysis. The particles were identified and classified by X-ray spectroscopy.

2. Analysis of histological section in SEM (Fig.1)

A first HES stained histological section was observed under an optical microscope (ZEISS Axio scope A.1). A second 5 µm thick unstained section was deposited on a carbon disc with a diameter of 25 mm. This section was intended for observation by scanning electron microscopy (JEOL JSM-6010PLUS / LV) equipped with an Oxford X-Max micro-analyzer 50 mm^2. The analysis of the particles was carried out under the following conditions: backscattered electrons; 20kV intensity; 12mm working distance; Spot size 72; Pressure 20 Pa; Magnification x500. At this magnification one field corresponds to 0.05 mm^2. For each sample, 5 fields were analyzed.

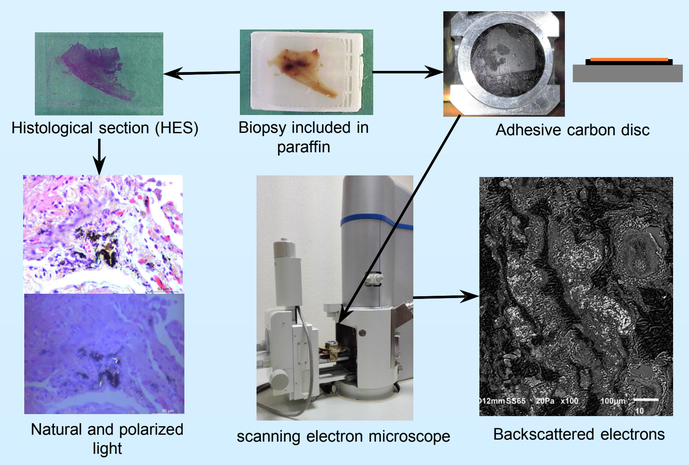

Figure 1: *In situ* mineralogical analysis in optical and electron microscopy from a paraffin-embedded biopsy.

III. Results

A. Results of the MA (Table 2)

Table 2 shows the result of the MA on the BAL fluids and on the paraffin embedded biopsies from the 10 sarcoidosis patients. For the BAL fluid, the ranking was based on the 39 individuals included in the study (one healthy subject was excluded for MA). For the biopsy, the dust level was calculated according to the average number of particles per field. The particles analyzed by EDX were classified according to their chemical composition.

Table 2: Results of the mineralogical analysis in electron microscopy. Level of particles load: + : less than 10 particles per field ; ++ : 10 to 50 particles per field ; +++ : 50 to 100 particles per field ; ++++ : more than 100 particles per field. P: phosphorus; Ca: calcium; Si: silicon; O: oxygen; Al: aluminum; Na: sodium; Fe: iron; Mg: magnesium; Ti: titanium; Ni: nickel; Cr: chromium; Cl: chlorine. The chemical composition of the analyzed particles is reported by indicating the elements detected on the EDX spectrum. Considering the suspected exposures, "SiO" Is interpreted as being silica, "PCa" as calcium phosphate probably of endogenous origin, "SiAl" as aluminosilicates, "PNa" as sodium phosphate, "TiO" as titanium oxide, "Compo Ti" as particles composed of titanium, "FeCrNi" and "FeCr" as steel, "TiNi" as titanium and nickel alloy, "CaO" as calcium oxides, "Cl" as chlorine particles.

PN	Sampling type	BAL Fluid MA	*In situ* MA	
			Level of particles load	Chemical distribution of the particles
1	EBUS/TBNA	1st Cr	++++	42% PCa ; 32% TiNi ; 22% FeCrNi
2	EBUS/TBNA	2nd Cr	++++	35% TiNi ; 25% PCa ; 24% FeCrNi ; 10% TiO
3	EBUS/TBNA	2nd FeCrNi ; SiMg	+++	40% PCa ; 23% FeCrNi ; 21% TiNi ; 11% compo Ti
4	EBUS/TBNA	4th Ti ; 5th SiO ; 3rd Al	++++	33% SiO ; 33% FeCrNi ; 33% PCa
5	EBUS/TBNA	After the 5th rank	++++	35% TiNi ; 22% SiO ; 18% SiAl ; 17% FeCrNi
6	BB	2nd SiO ; 3rd SiAl ; 5th FeCrNi	+	60% PCa ; 26% FeCrNi ; 11% TiNi
7	BB	After the 5th rank	+	33% FeO ; 33% FeCrNi ; 33% FeCr
8	BB	3rd FeCrNi ; 4th FeO	+	33% FeCrNi ; 33% Cl ; 33% FeCr
9	BB	4th Cr	++	17% PNa ; 17% CaO ; 17% FeO ; 17% SiAl ; 17% PCa ; 17% FeCrNi
10	BB	1st FeCrNi	+++	35% FeCrNi ; 32% PCa ; 16% CaO ; 10% FeO

B. Illustration of patients n°1 and 4 (Fig. 2 and 3)

Patient N°1 was a 41-year-old man of French origin, public relations worker, and suffering from stage 2-sarcoidosis. The mineralogical analysis of the BAL fluid revealed a high rate of chromium particles compared with the 19 healthy volunteers included in the MINASARC study. Fig. 2 shows the mineralogical analysis of the lymph node biopsy performed by endobronchial ultrasound trans-bronchial needle aspiration (EBUS/TBNA). Optical microscopy revealed the presence of opaque and birefringent particles. Electron microscopy showed the presence of numerous inorganic particles (TiNi and FeCrNi) gathered into a cluster.

Patient N°4 was a 29-year-old woman of French origin, non-smoker, esthetician manicurist and affected with stage 1-sarcoidosis. The mineralogical analysis of the BAL fluid revealed a high level of Sulfur compound particles and silica particles compared with the 19 healthy volunteers included in the MINASARC study. Fig. 3 shows the mineralogical analysis of the lymph node biopsy performed by EBUS/TBNA. Optical microscopy revealed the presence of opaque and birefringent particles. Electron microscopy showed the presence of numerous inorganic particles (SiO and FeCrNi).

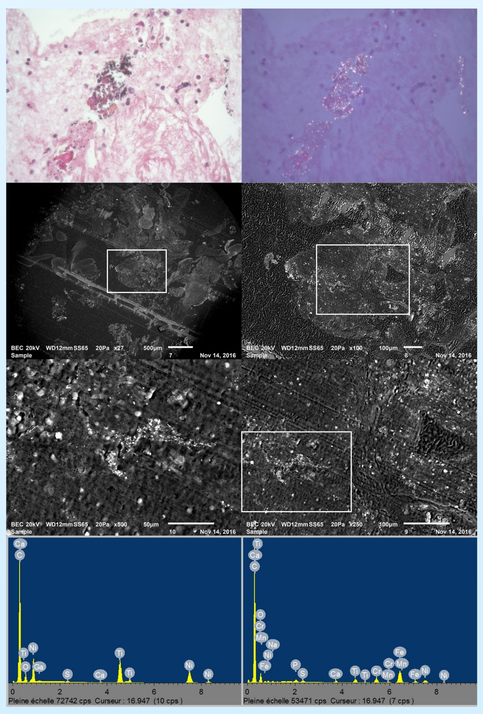

Figure 2: *In situ* mineralogical analysis in optical and electronic microscopy of the biopsy of patient n°1.

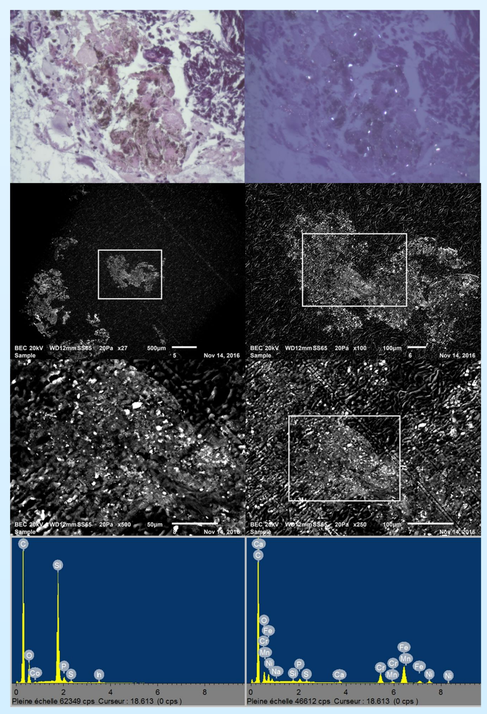

Figure 3 : *In situ* mineralogical analysis in optical and electronic microscopy of the biopsy of patient n°4.

IV. Discussion and conclusion

● MA-SEM on tissue sections is feasible and can give interesting results when compared with the MA of the BAL fluid and the patient's occupational and environmental exposure. In patient N°4, a manicurist, it identified silica particles in the lymph node and a high level of silicon oxides in the BAL fluid. As mentioned [1], these silica particles may arise from the powder used to polishing the nails before the methacrylate polymer application. Such an exposure may be considered to be involved in the granulomatosis affecting the patient. In patient N°10, a safety worker, steel particles were identified in the biopsy associated with a steel overload in the BAL fluid.This patient has worked as a plumber (less than one year). During this period, he carried out welding and drilling on metal products and paints. In this patient, exposure to metallic particles may be suspected of being a cause of granulomatosis in reaction to steel.

● For EBUS/TBNA cases, we identified FeCrNi and TiNi particles compatible with metal particles released from the needles used for TBNA, as already reported [2].

● The size of the biopsy is an important factor for the *in situ* MA. In order to have an adequate sensitivity of the analysis, it is necessary that the surface of the histological section be greater than 0.5 cm^2.

● The *in situ* MA with SEM that we performed neither allows us to analyze all the particles present on the histological section nor to detect nanoparticles. This is feasible with the development of an in situ analysis using the Laser Induce Breakdown Spectrometer (LIBS) [3].

● The *in situ* MA with SEM on paraffin embedded biopsies allows to determine the chemical distribution of particles observed in the preserved structures of tissues. It offers additional information on the patient's exposure to inorganic dusts and, for some patients, it helps to shift the diagnosis toward a mineral dust-induced granulomatosis.

● Calcium phosphate particles were observed in 7 out of 10 patients. This compound has a probable endogenous origin and could be related to the onset of the pathology as described for sarcoidosis [4,5].

Références : [1] Maxfield R and Howe HL. Epidemiol Report Series 97:8. Springfield, Il: Illinois Department of Public Health, November 1997. [2] Gounant et al. Chest 2011;139(1): 138-143. [3] Busser et al. ERS Milan 2017. [4] Demetriou ETW et al. J Bone Miner Res 2010;25(7):1695-1699. [5] Kimoto T. Internal Medicine 2003;42(11):1155.