Sarcoidosis and pulmonary dust: Mineralogical Analysis (MA) and Optical Microscopy (OM)

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I. Introduction

The building of the entity of sarcoidosis or Besnier-Boeck-Schaumann disease between 1888 and 1915 led to the concept of a systemic granulomatous disease affecting mainly the lungs and the lymphatic system, the cause of which remain unknown since then. Among the possible etiological factors of the disease (Newman & Newman, 2012), exposure to inorganic mineral dust especially silica, silicates and metals has been drawing more and more attention for the last fifteen years. To put this hypothesis to the test, the development of method allowing to assess the overload of inorganic particles in biological samples could help clarify their role in the onset of sarcoidosis. We present here the contribution of the digital image analysis to describe the particulate load in bronchoalveolar lavage (BAL). This is a retrospective study assessing the correlation between pulmonary dust load and sarcoidosis.

II. Material and methods

Population of sarcoidosis subjects

24 patients (15 men and 9 women) with a mean age of 43 years (min: 27 and max: 61), followed at the St Joseph St Luc Hospital with sarcoidosis (4 stage I, 7 stage II, 9 stage III and 4 stage IV), among them 2 Löfgren syndrome cases and 22 with histological evidence. For these patients, a MA of the BAL was requested to the mineralo-pathology laboratory of St Joseph St Luc Hospital of Lyon between 2005 and 2013.
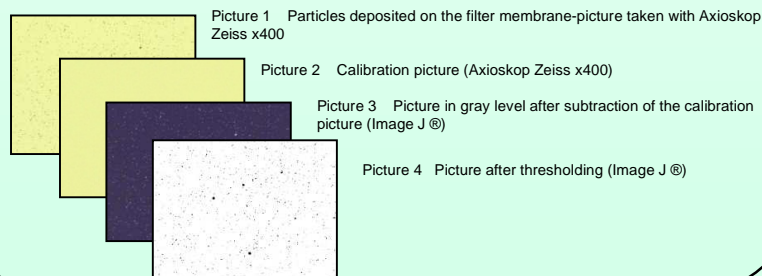
Population of "control" subjects

6 subjects with a MA of the BAL required for interstitial lung disease and performed in the mineralo-pathology laboratory of St Joseph St Luc Hospital of Lyon between 2005 and 2013. Their diseases were finally classified as not related to mineral dust exposure (1 post radiotherapy BOOP, 1 tuberculosis, 2 varicella, 1 metastatic micro-nodules, 1 bleomycine pneumonia).

Preparation of BALs

The BALs were prepared according to a method of digestion-filtration: the sample was digested with sodium hypochlorite in aqueous solution and after centrifugation and rinsing the solution was filtered through a MF-Millipore membrane with a 0.45 µm-pore diameter. Deposited on a slide, the membrane is made transparent in acetone vapors and then observed with an optical microscope Zeiss Axioskop 40 coupled to an AxioCam ICc3 camera.

Digital images analysis from BAL filtration

At a magnification of 400, three fields were randomly selected after checking the homogeneity of the entire filter and were photographed in natural light. The images were analyzed by the software ImageJ® (Abramoff, 2004). After subtraction of a calibration picture, the picture in gray level was segmented by thresholding in order to differentiate items of interest from the background. Particles having a size less than 3 pixels and particles in contact with the edge of the image were ignored. For each identified particle, the 2-dimensional area parameters and Feret's diameter were determined. The average values of the total particle number, the particle number whose size was less than 1µm, and the particle size were reported. The particle rate was expressed in the unit of "number of particles per ml of BAL". A "white" filter, prepared from the digestion-filtration of 20 ml of physiological salt solution instead of the BAL fluid appeared to contain a particles content of $6.9 \cdot 10^2$ per ml, $6.0 \cdot 10^2$ of which had a diameter less than 1 µm.

Picture 1   Particles deposited on the filter membrane-picture taken with Axioskop Zeiss x400

Picture 2   Calibration picture (Axioskop Zeiss x400)

Picture 3   Picture in gray level after subtraction of the calibration picture (Image J ®)

Picture 4   Picture after thresholding (Image J ®)

III. Results

Figures 1, 2 and 3 represents (in logarithmic scale, as boxplots) the distribution of: 1) the total number of particles per ml of BAL, 2) the particles number whose size is less than 1µm, 3) the particle size for each group. The geometric means are:

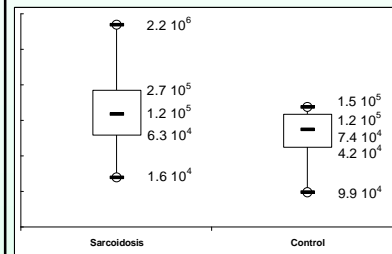|	Sarcoidosis	Control
Total numbers of particles per ml of BAL	$1.4 \cdot 10^5$	$5.9 \cdot 10^4$
Number of particles whose size is less than 1µm	$9.6 \cdot 10^4$	$4.5 \cdot 10^4$
Means Feret diameters (µm) of particles	0.99	0.93

Figure 1
Boxplot showing the distribution of the total number of particles per ml of BAL measured by image analysis for groups of patients and control subjects.
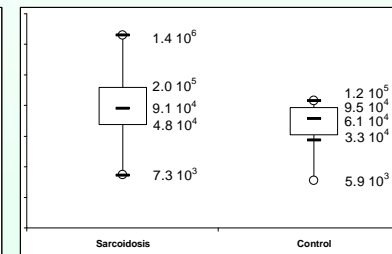

Figure 2
Boxplot showing the distribution of the number of particles whose size is less than 1µm per ml BAL measured by image analysis for groups of patients and control subjects.
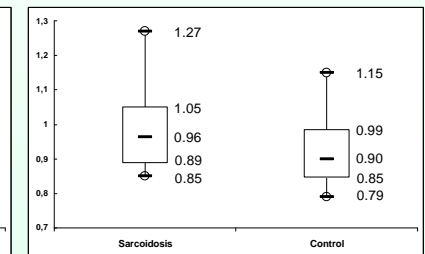

Figure 3
Boxplot showing the distribution of the Feret particle diameters (µm) of particles measured by image analysis for groups of patients and control subjects.

The dust load values were subjected to a logarithmic transformation before comparison. After checking the equality of variances with a Fischer test, a Student's t test was performed in the three cases (Excel ®). The differences between the two groups (sarcoidosis/controls) were not significant:

Total number of particles per ml of BAL: t*=1.72 (df=28) p=0.10

Number of particles whose size is less than 1µm: t*=1.49 (df=28) p=0.15

Particle size: t*=1.00 (ddl=28) p=0.35

IV. Discussion

The MA allows describing the following parameters of mineral particles deposited on a filter after digestion of a BAL: total number of particles per ml of BAL, number of particles whose diameter is less than 1µm, particle Feret diameter. The measurement made for this study varied so considerably that the difference between the group of the 24 sarcoidosis (globally greater dust load) and the group of the 6 control subjects can be attributed to the variability induced by the sampling process. The size of the groups was indeed insufficient, particularly that of the 6 control subjects. Moreover, the fact that the "control group" was not composed of healthy controls might have induced an increase in the measured values, even if their disease, with an etiology a priori unrelated to lung dust load, allowed to assimilate these patients to "controls". Finally, this optical analysis method does not allow us to observe the submicroscopic particles (<0.3 microns) and to determine their chemical composition. For that, electron microscopy coupled to a microanalysis is necessary. Some other studies showed that mineralogical analysis by optical microscopy is useful but not sufficient to describe a mineral overload (Catinon et al., 2014).

Even though patients had not been submitted to a systematic environmental and occupational questionnaire, we could record several dusty activities: sweeper in foundry, boilermaker, electrician (2), gardener, mechanic, housekeeper (3), construction worker, carpenter. The major occupational and health surveys conducted in general population tend to underestimate the sanitary risks of "dusts", particularly the risk "related to crystalline silica" (Cavalin et al., 2013). A specific "whole life" questionnaire would be highly useful.

V. Conclusion

Mineralogical analysis with quantification of non-fibrous particles by digital image processing is an innovative method to describe and screen mineral overloads. The number of subjects included in the present retrospective study either as patients or controls proved however to be too low to produce significant statistical differences between the measured particle loads. Moreover, the "imperfect" control subjects of this study have leaded us to think of conceiving a pilot prospective study with pathological and healthy subjects. MINASARC01, a case-control study which is currently conducted, aims at comparing blinded data drawn on the one hand from the mineralogical analysis of BALs and on the other hand from an environmental and occupational questionnaire submitted to 20 healthy subjects and 20 patients with sarcoidosis. MINASARC01 will resort to electron microscopy with micro-analysis to determine the nature of the particles observed on filter. This study is conceived, by a multidisciplinary working group including the SILICOSIS team (ERC Advanced Grant) led by Paul-André Rosental at the Center for European Studies, Sciences Po Paris and the Sarcoidosis Group of the French Society of Pneumology (SPLF). It brings together pulmonologists, physicians, pathologists, sociologists, historians of science and mineralogists.

References :   Newman KL. and Newman LS. Curr Opin Allergy Clin Immunol 2012; 12: 145-50.   Abramoff MD. J Biophotonics Intern.2004; 11: 36-42   Catinon M. et al. Sarcoidosis Vasc Dif (in press)   Cavalin C. et al. Environnement, Risques et Santé 2013; 12: 352-8