Metal load assessment in patient pulmonary lavages: towards a comprehensive mineralogical analysis including the nano-sized fraction

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Word count: 6418.

Abstract

Mineralogical analyses of clinical samples have been proved useful to identify causal relationship between exposure to airborne particles and pulmonary diseases. The most striking example is asbestosis where the assessment of asbestos bodies in patient lung samples has allowed defining values specific of pathologies. However this type of analyses only considers the micro-sized fraction of the particles, neglecting the specific impact of nano-sized particles which have been otherwise shown to be reactive and able to induce biological effects. Similarly, in nanotoxicology, the mineralogical analysis of pulmonary fluids could be used as an indicator of exposure to inhaled nanoparticles and could help investigations on the relationship between exposure to these nanoparticles and lung diseases. We designed this study first to demonstrate the technical feasibility of this approach, then to get a clear picture of the metals present, and in what form, in patient lungs and finally to determine if indeed it is worth investigating separately the micro, sub-micro and nano fractions. Broncho-alveolar lavages were recovered from 100 patients suffering from interstitial lung diseases. A protocol was specifically developed to isolate 3 fractions containing respectively microparticles, sub-microparticles and nanoparticles with ions. The metal content in each fraction was qualitatively and quantitatively characterized. Results showed significant differences between the 3 fractions in terms of metal load confirming that the separate analysis of the fractions is relevant. It also means that the assessment of the micro-sized fraction alone, as commonly done in clinical practice, only gives a partial view of the mineralogical analysis.

Keywords

Mineralogical analysis; Interstitial lung diseases; Inhaled particles; Nanotoxicology; Metal load.

Introduction

Lung pathologies induced by inhaled biopersistent mineral particles have been described for a long time now. In particular, pneumoconioses are a group of lung diseases caused by the inhalation of certain dusts (such as asbestos fibers, silica, beryllium, aluminum, bauxite, antimony, barium, graphite, iron, kaolin, mica, talc, etc.) and the associated lung tissue's reaction to the mineral dust (National Institute for Occupational Safety and Health, n.d.). It is commonly acknowledged that pneumoconioses are occupational diseases (National Institute for Occupational Safety and Health, n.d.). The most documented and dramatic examples are asbestosis or silicosis. But it has been suggested that other lung pathologies could be caused by the inhalation of mineral particles such as idiopathic fibrosis or sarcoidosis (Hubbard, 2001; Iqbal et al., 2008; Rafnsson et al., 1998; Taskar and Coultas, 2008; Vincent et al., 2004; Vincent and Lievre, 2002). Furthermore, the impact of ultrafine particles on human health has been well described (Brunekreef and Holgate, 2002; Rinaldo et al., 2015).

In this context, to help reduce health risks, different techniques have been used to measure exposure to nanomaterials in the workplace (ISO/TS 12901-1:2012, 2012). But this external dose is not sufficient to determine a health risk as many other parameters enter into the equation such as the breathing pattern of each worker or their position relative to the emitting source. It is therefore necessary to assess the internal dose. This can be done through the bio-monitoring of chemical elements, especially metals. Whereas air monitoring indicates the possibility and range of exposure by inhalation, biological monitoring gives an indication of the absorbed amount of biopersistent metals in the individual worker (Miksche and Lewalter, 1997). It is mainly used in a context of occupational medicine as a tool to prevent workers from being exposed to high concentrations of toxic substances, thus reducing the health risk (Beattie et al., 2017; Gube et al., 2013; Riihimäki and Aitio, 2012). We may wonder if such bio-monitoring of biopersistent metallic particles could have such relevance in a clinical

context, *i.e.* regarding the analysis of samples from patients suffering from a lung disease. Indeed, toxicity often results from the accumulation and biopersistence of particles. Despite clearance mechanisms or translocation, about 10 to 20% of insoluble (or poorly soluble) particles remain in the lung where they can trigger adverse effects (Rinaldo et al., 2015). It intersects with the concept of mineralogical analyses of which the usefulness has been perfectly illustrated in the case of asbestosis where the assessment of asbestos bodies (AB) in patient lung tissues or in broncho-alveolar lavage (BAL) fluids has allowed defining threshold values specific of pathologies (1000 AB/g in bronchial cancer, 6000 AB/g in mesothelioma and more than 100000 AB/g in asbestos fibrosis against 67 AB/g in healthy control) (De Vuyst et al., 1998).

It has been proposed that mineralogical analyses could be extended to nanotoxicology issues especially to get new insights in the role of inhaled biopersistent nanoparticles in the etiology or development of some respiratory diseases. For instance, it has been suggested that carbon nanotubes could exhibit a toxicity similar to that of asbestos fibers due to a resemblance in shape. And recently, through the analysis of BAL from asthmatic children, a study has evidenced that carbon nanotubes can be accumulated in lungs and thus potentially contribute to the pathologic condition of the subjects (Kolosnjaj-Tabi et al., 2015). However, the development of mineralogical analyses in nanotoxicology is still limited. A first reason is the complexity of technical challenges as previously reviewed (Bitounis et al., 2016). The main hurdle lies in the change of scale of the sought particles, requiring adapted equipment for nanoparticle quantification. A second limiting factor lies in the fact that the samples are usually analyzed globally, *i.e.* without separating their constitutive fractions. In other words, with the current methodology used for asbestos-like mineralogical analysis (De Vuyst et al., 1998) only the micrometric particles can be assessed and neither the nano-sized particles nor the soluble part (ions) of the metal load are considered while these two fractions are able to

trigger biological effects. Indeed, nanoparticles are recognized to be more reactive and able to induce more adverse health and cellular effects than larger particles of the same composition (Cena et al., 2014).

Many studies investigating the relationship between exposure to airborne nanoparticles and lung diseases are available in the literature. Experimental studies both *in vitro* and *in vivo* (animal models) have shown that exposure to nanoparticles could induce oxidative stress, pro-inflammatory effects and lead to clinical pictures similar to emphysema or fibrosis (TiO_2) or mesothelioma (carbon nanotubes) (Andujar et al., 2009). But in humans, very few studies are available. Song *et al.* (Song et al., 2011, 2009) found silica nanoparticles in clinical samples from 7 patients suffering from lung injuries after an occupational exposure. But as these patients were exposed to other toxic substances than dust and polyacrylate nanoparticles no firm conclusion could be reached. Another study (Andujar et al., 2009) had reported that pulmonary injuries were more severe in welders than in unexposed people suggesting that nanoparticles present in welding fumes could be responsible, at least in part, for the pulmonary inflammation. But, once again this study was limited to the description of few clinical cases and did not have a significant statistical power. It was also restricted to a target population and conclusions can hardly be extrapolated. In the literature, the investigation of nanoparticles in patients' samples is rare and when it exists it is limited to electron microscopy observations that do not allow a complete physicochemical characterization of the nanoparticles. However, in the nanotoxicology field, an exhaustive characterization of nanoparticle physico-chemical features is needed, as recommended within the ISO technical report 13014 and recently underlined by American Chemical Society Editors (ISO/TR 13014:2012, 2012; Mulvaney et al., 2016).

We have recently explained in details (Forest et al., 2017) how, in our opinion, mineralogical analysis of patient samples associated to standard *in vitro/in vivo* nanotoxicology assays could

bring new avenues of research starting from a clinical reality. We strongly believe that the mineralogical analysis of metal load extracted from pulmonary fluids could be used as an indicator of exposure to nanoparticles and could contribute to the assessment of potential causal links between the presence of inhaled biopersistent nanoparticles in the lung and respiratory diseases. Of course, this approach has to face technical challenges and requires specific protocols to be established and detection methods to be adapted. This is why we designed this study, first to demonstrate the technical feasibility of this approach, then to get a clear picture of the metal load present in patient lungs and finally to determine if indeed it is worth investigating separately the micro, sub-micro and nano/ion fractions of a BAL clinical sample. Concretely, the aim of this exploratory study was thus to characterize both qualitatively and quantitatively the chemical element content of BAL from 100 patients suffering from interstitial lung diseases and needing this invasive exam for clinical purpose. This investigation included the assessment of 3 fractions consisting of microparticles, sub-microparticles and nanoparticles/ions which were separated by a protocol specifically developed for this study.

Materials and Methods

Patients

During 2 years, 100 patients from the Chest diseases and thoracic oncology Department of the University Hospital of Saint-Etienne were included in this prospective, monocentric and exploratory study (NanoPI, ClinicalTrials.gov Identifier: NCT02549248). Patients were presenting a clinical picture of diffuse interstitial lung disease and needed a bronchoscopy associated with a BAL (*i.e.* this exam was not performed on purpose for this study). Patients were informed about this study and had to give their written consent to participate in. Our protocol was in accordance with ethical principles defined by the World Medical Association

declaration of Helsinki and subsequent amendments. It was approved by an ethics committee (Comité de Protection des Personnes, Sud-Est I) and by the French agency regulating biomedical research (Agence Nationale de Sécurité du Médicament et des produits de santé, ANSM).

Criteria for the inclusion in the study were: i) patients with an interstitial lung disease assessed on clinical signs and CT scan, requiring a flexible bronchoscopy associated with a BAL (for instance for patients suffering from sarcoidosis, idiopathic pulmonary fibrosis, hypersensitivity pneumonitis, infectious or cancerous interstitial diseases and interstitial diseases caused by drug reactions), ii) patients older than 18, iii) patients who had given their voluntary, informed and written consent, iv) patients having a social insurance or beneficiary (mandatory for any French clinical study). Exclusion criteria were: i) patients who had not given their consent, ii) when flexible bronchoscopy or BAL was not possible, iii) patients under legal protection or pregnant women, iv) patients with contagious disease (HIV infection, tuberculosis, viral hepatitis) for safety reason.

Broncho-alveolar lavages

Broncho-alveolar lavages were performed following regular practice in the Chest diseases and thoracic oncology Department of the University Hospital of Saint-Etienne. Briefly, 50 mL of a warmed saline solution were injected in the selected area of lung and were slowly aspirated, the collected sample constituting the bronchial wash (BW). Then 2 to 4 additional 50 mL of warmed saline solution were injected and aspirated successively, the collected sample representing the BAL strictly speaking. All samples were collected into plastic tubes without any fixative agent. They were immediately transferred to the Histology-Cytology Department for standard cytological analysis. After this analysis, if there was still enough volume of sample 5 mL of BW and 20 mL of BAL were dedicated to the present study. An equivalent

volume of sodium hypochlorite was added to the samples that were then stored at 4°C until use.

Sample pre-treatment: separation of the micro, sub-micro and nano/ion fractions

BAL and BW underwent the same process consisting of a 2 step-centrifugation as illustrated by Figure 1 and as described in details below. The first centrifugation was short and allowed to separate the fraction containing microparticles from the fraction containing sub-micro and nano-sized particles and potentially soluble metals (ions). The second centrifugation, longer than the first one, allowed separating sub-microparticles from nanoparticles and ions.

Step 1: isolation of the "micro" fraction – Samples were vortexed and sonicated using a Branson sonicator and Cup Horn (70%, 2 min at room temperature). 1 mL of sample was transferred into a 2 mL Protein LoBind tube (Eppendorf®). 0.5 mL of an extraction cushion consisting of a glycerol 75%v/v, NaOH 0.001 M 25%v/v solution was added to the samples. Because of their difference of density the sample and the glycerol solution did not mixed. The tubes were centrifuged 10 min at 0°C, 1600g (4000 rpm) (Thermo Fisher Scientific Heraeus megafuge 16R). Tubes were then punctured at the level of the 0.25 mL mark with a needle (22G x $1^{1/2}$ Terumo©) mounted on a 2 mL syringe and the supernatant was aspirated and transferred into a new Protein LoBind tube, this part contained the sub-microparticles, nanoparticles and ions (≈ 1.25 mL). The pellet containing the microparticles was resuspended with 0.75 mL of NaOH 0.001 M and was transferred into a new Protein LoBind tube and stored at 4°C until analysis.

Step 2: separation of the "sub-micro" fraction from nano and ions – 0.5 mL of an extraction cushion (glycerol 75%v/v, NaOH 0.001 M 25%v/v) was added to the tube containing the sub-microparticles, nanoparticles and ions fractions obtained from step 1. Tubes were centrifuged during 3h30 at 0°C, 2500 g (5000 rpm). Tubes were then punctured at the 0.5 mL mark with a

needle (22G x $1^{1/2}$ Terumo©) mounted on a 2 mL syringe and the supernatant was slowly aspirated and transferred into a new Protein LoBind tube, this part contained nanoparticles and ions. The pellet containing the sub-microparticles was resuspended with 0.6 mL of NaOH 0.001 M and was transferred into a new Protein LoBind tube and stored at 4°C until analysis. To assess the background noise a "blank" sample consisting of distilled water underwent the same protocol.

Sample analysis

All fractions (micro, sub-micro, nano/ion) were analyzed using both dynamic light scattering (DLS) method (Nanozetasizer®, Malvern Instrument) and inductively coupled plasma atomic emission spectroscopy (ICP-AES, Jobin-Yvon JY138 Ultrace). DLS allowed verifying that size fractionation was successful. ICP-AES allowed determining for each metal the quantity of matter expressed in parts-per-billion (ppb). We focused our investigation on the following metals: aluminum (Al), beryllium (Be), cobalt (Co), chromium (Cr), copper (Cu), iron (Fe), nickel (Ni), silicon (Si), titanium (Ti), tungsten (W), zinc (Zn) and zirconium (Zr). Samples from the micro fractions were also observed using optical microscope and some samples of interest from the sub-micro fractions were observed using electron microscope.

Scanning electron microscopy images and EDX analysis

Specimen holders were coated with carbon tape and sample droplets (~4 µL) were deposited and left to dry at 200°C for about 5 min protected from dust. Images were obtained using the angular selective back-scatter detector operated between 10 and 20 keV and the in-lens secondary electron detector operated at 1 keV, at a working distance of ~4 mm (Zeiss SUPRA^{TM}55VP equipped with a Gemini column and an EDX Oxford X-Max^{N}80 detector). Acquisition of EDX spectra were performed for the qualitative analysis of the particles.

ICP-AES analysis

While the sub-micro and nano/ion fractions could be directly analyzed with ICP-AES (0.5 mL of samples has just to be transferred into 16 mL glass tubes and added with 9.5 mL of PBS), the micro fraction needed an additional pre-treatment of mineralization (acid attack). To that purpose, 0.4 mL of sample was added in a Savillex™ tube containing 3.45 mL of HCl 37%. After a 3h incubation, 0.15 mL of H_2O_2 30% was added. After another 3h incubation, 1 mL of concentrated HNO_3 (>65%) was added and placed overnight at 80°C to evaporate. 10 mL of HCl 2M were finally added before analysis.

To assess the background noise a "blank" sample consisting of 10 mL of distilled water followed the same protocol and was analyzed.

The ICP-AES analysis allowed quantifying chemical species in ppb, *i.e.* as ng/mL. Values lower than the limit of detection (LoD) were considered as not detectable. The LoD were calculated based on literature reference (Armbruster and Pry, 2008). We then assessed the number of patients for which the value was higher than the LoD.

Results

1. Technical feasibility of the approach: isolation of the micro, sub-micro and nano/ion fractions of pulmonary lavages and assessment of the metal load

1.1. Quality control of the size fractionation

The DLS analysis allowed verifying the efficiency of size fractionation. Figure 2 provides an example representative of all samples. Typically, the peak for the micro fraction was around 1 µm, that of the sub-micro fraction was ranged between 100 nm and 1 µm and that of the nano/ion fraction was under 100 nm. No nanoparticles were found in the micro fraction and conversely no microparticles were observed in the sub-micro and nano/ion fractions, confirming the efficiency of our protocol of size fractionation.

1.2. Quantitative analysis of the metal load in the different fractions

The results from the ICP-AES analysis of the micro, sub-micro and nano/ion fractions of the BW and BAL samples are reported in Table 1.

1.3. Observation of particles with electron microscopy

Figure 3 illustrates particles observed with electron microscope (gold standard technique for mineralogical analysis of pulmonary lavages) in the micro and sub-micro fractions for a representative patient. Energy-dispersive X-ray (EDX) spectroscopy allowed verifying the chemical nature of the metals. The observations were consistent with results from the DLS and ICP-AES analyses.

2. Detailed analysis of the results of the mineralogical analysis of patient pulmonary lavages: relevance of the investigation of the micro, sub-micro and nano/ion fractions

Results from the ICP-AES analysis can be interpreted depending on different points of view: the patients, the type of sample (BW *versus* BAL), the type of fraction (micro, sub-micro, nano/ion) and the metals considered.

2.1. Metal load in pulmonary lavages from a 100 patient cohort:

Figure 4 reports the number of patients exhibiting a concentration in metals higher than the limit of detection in the micro, sub-micro, and nano/ion fractions of the BW. The same representation for BAL is reported in Supplementary data (Figure S1).

This type of representation gives a general overview of the metal load that can be mostly found in patient lung samples and their relative occurrence. Different profiles clearly appeared depending on the metal and the fraction considered. Observations were quite coherent, for instance, Si particles which are known to be highly insoluble were found in the micro and

sub-micro fractions, never in the nano/ion fraction. On the contrary, highly soluble particles such as Cr were mostly found in the sub-micro and nano/ion fractions, and almost never in the micro fraction.

2.2. Comparison of the metal load in BAL and BW:

To consider the sample perspective and determine if the BW was representative of the BAL, we investigated when BW and BAL analyses gave similar results in terms of number of patients exhibiting metal concentrations higher than the limit of detection. For that purpose, we arbitrarily chose a cutoff of 10 patients (10% of our initial population of 100 patients). Results are reported in Supplementary data (Table S1).

To the question "is BW representative of BAL?", the answer tends to be "yes" as metals mostly found in BW and BAL are the same with however some exceptions preferentially found only in one type of sample. It globally suggests that BW and BAL can give similar results in the context of mineralogical analyses of the micro, sub-micro and nano/ion fractions. However, when comparing the metal concentration patient by patient for each fraction, no correlations clearly appeared between BW and BAL as reported in Supplementary data (Table S2).

2.3. Comparison of the metal load in the micro, sub-micro and nano/ion fractions:

Then, we considered the results from the fractions angle of view. To determine if the metals were similarly found in the micro, sub-micro and nano/ion fractions of BW or BAL samples, we investigated when the 3 fractions gave similar results in terms of number of patients exhibiting metal concentrations higher than the limit of detection. For that purpose, we arbitrarily choose a cutoff of 10 patients (10% of our initial population of 100 patients). Results are summarized in Table 2.

From this comparison, no obvious correlation appeared between the micro, sub-micro and nano/ion fractions for all the metals, whatever the type of sample. This was confirmed by further statistical analyses, patient by patient, as reported in Supplementary data (Table S3). It suggests that the analysis of the 3 fractions is relevant for mineralogical analyses. Moreover, it highlights that if we consider only the micro fraction, as it is commonly done, we only get a partial vision of the analysis.

2.4. Metals of exogenous origin mostly found in pulmonary samples:

Finally, we considered the results with respect to the metals mostly found in lung samples. From Table S1 and Table 2, it clearly appeared that Al is the only metal from a necessarily exogenous origin to be found in all types of samples (BW and BAL) and all fractions (micro, sub-micro and nano/ion). Similarly, Fe and Zn which can be of endogenous origin are present in all types of samples and fractions. Si, found both in BW and BAL, was present in micro and sub-micro fractions but not in nano/ion, which seems quite consistent with the poor solubility of this metal. On the contrary, Cr found both in BW and BAL was rather found in the sub-micro and nano/ion fractions, which is quite logical in view of its quite high solubility. The metals of certain exogenous origin mostly found in both type of samples and either type of fractions were Al, Si and Cr as compared in Figure 5.

Discussion

Correlations have been demonstrated between exposure to fine particulate matter and lung pathologies (Brunekreef and Holgate, 2002; Pope III C et al., 2002; Rinaldo et al., 2015). When inhaled particles are not efficiently cleared by chemical dissolution or physical translocation they can accumulated in the lungs where they can induce adverse effects (in particular oxidative stress or inflammation). This can provide a micro-environment favorable

to the development of respiratory pathologies. To gain insight into this issue, it could be interesting to start from a realistic situation and thus characterize particles present in clinical samples, *i.e.* in patient pulmonary lavages. By identifying the chemical nature, size and dose of biopersistent particles present in a target tissue, it could allow toxicologists to focus their efforts on particles of interest for mechanistic studies. However, as observed in the literature, this mineralogical analysis approach is far from being systematical. In addition, for nanoparticle detection, the gold standard technique remains electron microscopy, either scanning (SEM) or transmission electron microscopy (TEM), at best EDX to characterize the chemical composition of the nanoparticles (Dumortier et al., 1994; Gatti, 2004; Gatti and Rivasi, 2002; Song et al., 2009; Wu et al., 2010). But these techniques are quite demanding and time-consuming. It is one of the reasons why such studies are limited to a small number of patients (usually in the order of tens) and cannot reach statistical power. Furthermore, almost never is a complete physicochemical characterization carried out.

In the present paper, we proposed a mineralogical analysis of BAL and BW from 100 patients suffering from interstitial lung disease. To get a complete overview we established a protocol allowing to isolate and separately analyze the micro, sub-micro and nano/ion fractions. We used DLS and ICP-AES techniques allowing high-throughput analyses. In addition, we validated our observations on some samples of interest using the gold standard technique, *i.e.* electron microscopy coupled to an EDX analysis. First, we clearly demonstrated the technical feasibility of the proposed approach. Then, this observational study allowed us to draw a picture of the metals mostly found in the micro, sub-micro and nano/ion fractions of patient pulmonary lavages. We reached the following main conclusions: i) with a few exceptions, the analysis of BW and that of BAL gives similar results, ii) no correlation was found between the micro, sub-micro and nano/ion fractions regarding their metal content.

Please note that caution should be taken in the interpretation of results regarding some metals because of their origin. Indeed, some metals such as Fe and Zn can possibly come from an endogenous source and therefore their content is difficult to interpret. However, the other metals studied here are obviously from an exogenous origin.

Except for some minor discrepancies, the metal content of BW and that of BAL were quite similar whatever the fraction considered. We used these types of samples as they are considered to be the gold standards for clinical diagnosis and it is well admitted that they are representative of the lung content. However, it is perfectly possible to extend this analysis to other types of samples with the necessary adaptations due to the different biological matrices. In particular, samples obtained by less invasive procedures, as in the context of occupational medicine could be used, such as urine, blood, induced sputum and potentially exhaled breath condensate (ECB) (Hoffmeyer et al., 2012; Hulo et al., 2014; Riihimäki and Aitio, 2012). Tissue biopsies can also be used but due to their small size and a sampling issue they could be less representative of the lung metal content. However, a study comparing the metal content between BAL and that of lung tissue samples has found a positive correlation (Dumortier et al., 1994). Furthermore, solid tissues are technically more difficult to handle and adapted protocols should be developed. Nevertheless, Takada *et al.* have proposed an interesting approach with the use of electron probe microanalyzer with wavelength dispersive spectrometer for solid tissue analysis (Takada et al., 2014).

Concerning the comparison of the metal content in the different fractions, no clear correlation appeared between the micro, sub-micro and nano/ion fractions underlining the relevance to study the 3 fractions separately. Otherwise, we would get only a partial view of the mineralogical analysis. This approach is consistent with that adopted by Cena *et al.* (Cena et al., 2014) for the characterization of welding fumes exposure. It was based on the observation that welders are exposed to high concentrations of nanoparticles and that current studies

investigate exposure to welding fumes without differentiating between nanoparticles and larger particles, whereas welding fume nanoparticles have been found to be more toxic at the cellular level and to generate more reactive oxygen species (ROS) when compared to larger particles. Thus, they developed a method to collect, recover, and analyze Cr, Mn and Ni nanoparticles (metals that have been linked with severe health outcomes) generated during welding, separately from larger particles. Similarly, Riihimäki *et al.* (Riihimäki et al., 2008) highlighted the impact of the particle size on toxicity: while aluminum metal and aluminum oxide are practically insoluble in water and are thus presumably poorly absorbed into living organisms, oxidized aluminum, contained in ultrafine particles, behaves differently, since some aluminum of the welding fume is rapidly taken up by the welder's lungs. It argues once again for the study of the finest fraction separately from its larger counterpart. This conclusion is in agreement with one of our previous nanotoxicology study where we demonstrated that the agglomerates size of boehmite nanoparticles had a major impact on their toxicity, highlighting the need to study not only raw industrial powders containing nanoparticles but also the ultrafine fractions representative of respirable particles (Forest et al., 2014). Also, still in relation with particle size, nanoparticles, unlike larger particles, can cross biological barriers. Therefore, while the respiratory tract is considered as the primary target organ, inhaled nanoparticles can translocate and reach and affect distant organs (Riihimäki and Aitio, 2012). In this context, a recent study has demonstrated that environmental nanoparticles were significantly over-expressed in acute myeloid leukemia, suggesting their contribution to the development of this disease (Visani et al., 2016). Even if such relationship remains to be proven by further studies, it underlines the relevance to investigate the presence of biopersistent nanoparticles in clinical samples.

Finally, we observed that the metals of exogenous origin mainly found in the patient pulmonary lavages were Al, Cr and Si. All these metals are known for their toxic potential.

Aluminum, preferentially found in the micro fraction of our samples, has been shown to induce adverse human health effects. Regarding lungs, pneumoconiosis (aluminosis), asthma, diffuse parenchymal lung disease, interstitial inflammation and fibrosis have been described. Al may also affect central nervous system, bones and hematopoiesis. Local subcutaneous or intramuscular inflammation may also be observed after injection of vaccines containing aluminum adjuvants (Riihimäki and Aitio, 2012; Wesdock and Arnold, 2014). The International Agency for Research on Cancer has stated that: "Occupational exposures during aluminum production cause cancer of bladder, and of the lung… Occupational exposures during aluminum production are carcinogenic to humans (Group 1)" (IARC, 2012, 2010). Chromium was preferentially found in the sub-micro and nano/ion fractions of our samples. Its toxicity, especially of hexavalent chromium (Cr(VI)), on various organs has already been extensively described (Baruthio, 1992). The main target organs for Cr(VI) toxicity are the skin (skin lesion, dermatitis or sensitization), the respiratory tract (irritation, bronchitis, asthma and cancer) and the kidneys (proximal tubular nephropathy). Unlike Cr(III), Cr(VI) is able to cross cell membranes, bind to intracellular proteins and induce DNA damages (Cena et al., 2014; Miksche and Lewalter, 1997). Silica was preferentially found in the micro and sub-micro fractions of our samples. Silica exposure has been associated with several disorders: silicosis (which is recognized as one of the most important occupational diseases worldwide), infections (tuberculosis, mycobacterial, fungal or bacterial lung infections), chronic obstructive pulmonary disease, malignant diseases (lung cancer but also gastric, esophageal and several other types), autoimmune diseases (such as scleroderma or rheumatoid arthritis), and renal diseases (Leung et al., 2012). At a cellular level, these metals share general mechanisms, in particular the induction of inflammation and the production of ROS have been well documented. The subsequent damages (DNA, redox imbalance) lead to carcinogenic effects (Kawasaki, 2015; Rehman et al., 2017).

Conclusion

To get a comprehensive mineralogical analysis of clinical samples it is mandatory to consider all their constitutive parts, *i.e.* the micro, sub-micro and nano/ion fractions. Performing a global analysis could lead at best to a partial view and at worst to misleading conclusions. This type of analysis is promising as it could open new perspectives in our understanding of some causes, or at least contribution, of inhaled biopersistent particles to respiratory diseases. Obviously further studies are necessary and the next step is to confront these first results to patients' clinical data. Indeed, a more detailed analysis is required to investigate potential correlations between the nature of the metals found in patient bronchial samples and environmental or occupational exposure factors. Also, relationships with the diagnosed pathologies will be investigated, potentially allowing improving our knowledge on the etiology of some diseases and consequently improving their prevention and cure.

Acknowledgements

The authors would like to thank the Fond de recherche en Santé Respiratoire de la Société de Pneumologie de Langue Française (NanoPI grant), the Canceropôle CLARA (Expo-Nano grant) and Santé-Environnement Rhône-Alpes (EnvitéRA) for the financial support.

Disclosure of interest

The authors report no conflicts of interest.

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Figure captions:

Figure 1 - Schematic representation of the size fractionation protocol of BAL or BW sample into micro, sub-micro and nano/ion fractions.

Figure 2 – DLS analysis of the micro (A), sub-micro (B) and nano/ion (C) fractions of the BAL from patient n°3.

Figure 3 – Electron microscope observation and energy-dispersive X-ray spectroscopy of the micro (A) and sub-micro (B) fractions of the BW sample from patient n°61.

Figure 4 – Number of patients with a metal concentration higher than the limit of detection (LoD) in BW for the micro, sub-micro and nano/ion fractions.

Figure 5 – Comparison of the Al (A), Cr (B) and Si (C) concentrations in the micro, sub-micro and nano/ion fractions of BW and BAL. The median was calculated when at least 10 patients exhibited a metal concentration higher than the limit of detection (the number of patients is indicated in brackets).

Figure 1

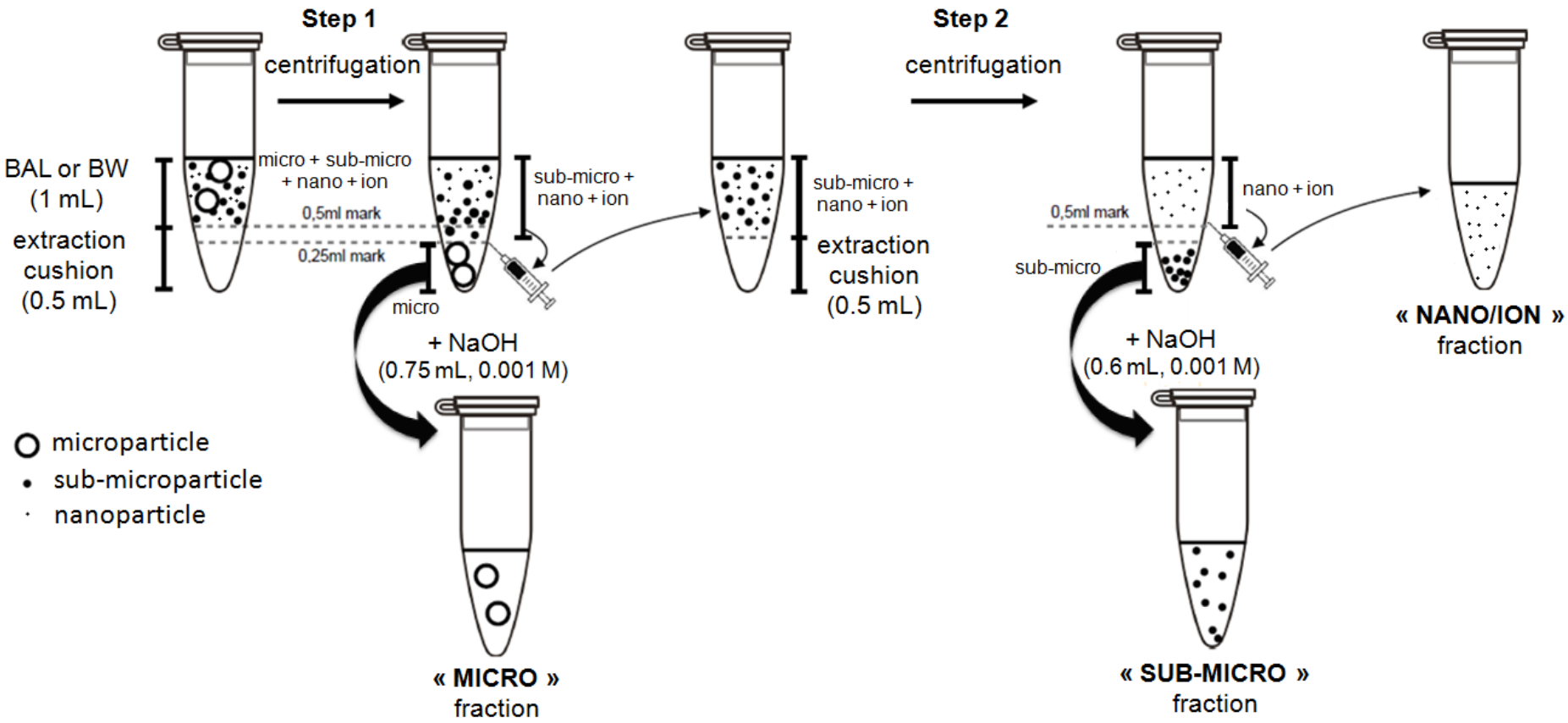
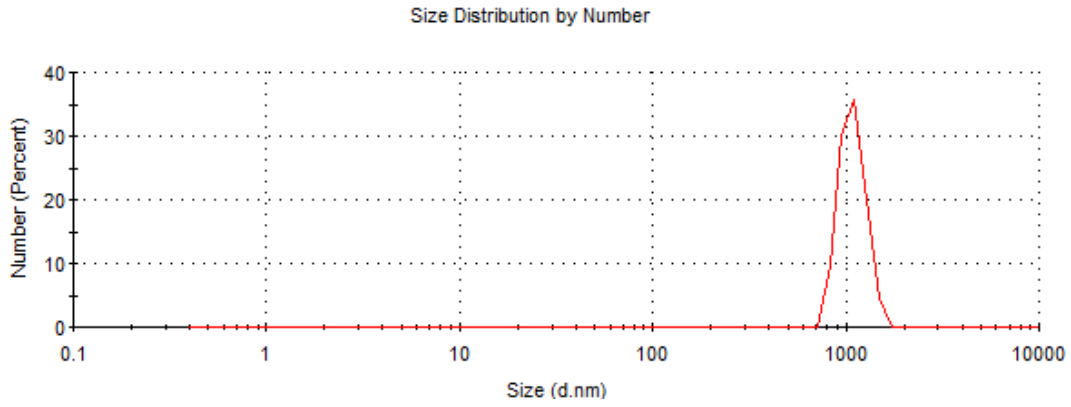
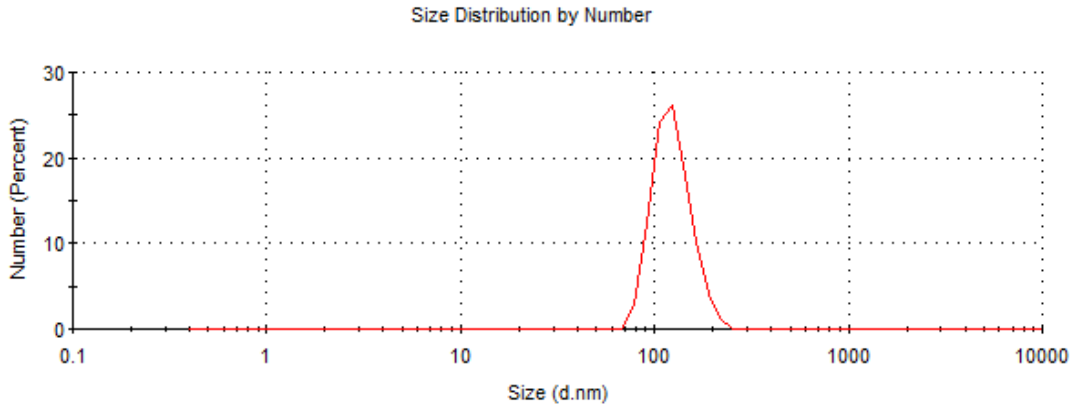

Step 1
centrifugation
BAL or BW
(1 mL)
extraction
cushion
(0.5 mL)
micro + sub-micro
+ nano + ion
0,5ml mark
0,25ml mark
micro
+ NaOH
(0.75 mL, 0.001 M)
« MICRO »
fraction
sub-micro +
nano + ion
sub-micro +
nano + ion
extraction
cushion
(0.5 mL)
Step 2
centrifugation
0,5ml mark
sub-micro
nano + ion
+ NaOH
(0.6 mL, 0.001 M)
« SUB-MICRO »
fraction
« NANO/ION »
fraction
microparticle
sub-microparticle
nanoparticle

Figure 2

A)


Size Distribution by Number
Number (Percent)
0
10
20
30
40
0.1
1
10
100
1000
10000
Size (d.nm)

B)


Size Distribution by Number
Number (Percent)
0
10
20
30
0.1
1
10
100
1000
10000
Size (d.nm)

C)

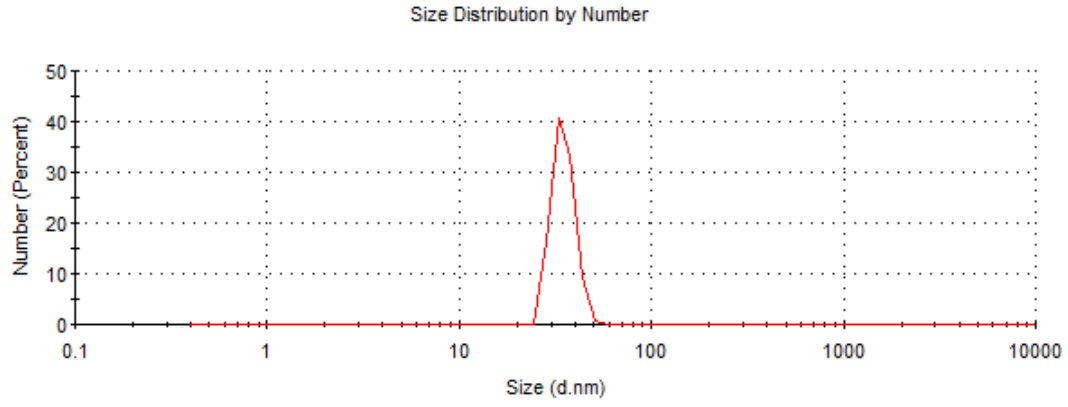

Size Distribution by Number
Number (Percent)
0
10
20
30
40
50
0.1
1
10
100
1000
10000
Size (d.nm)

Figure 3

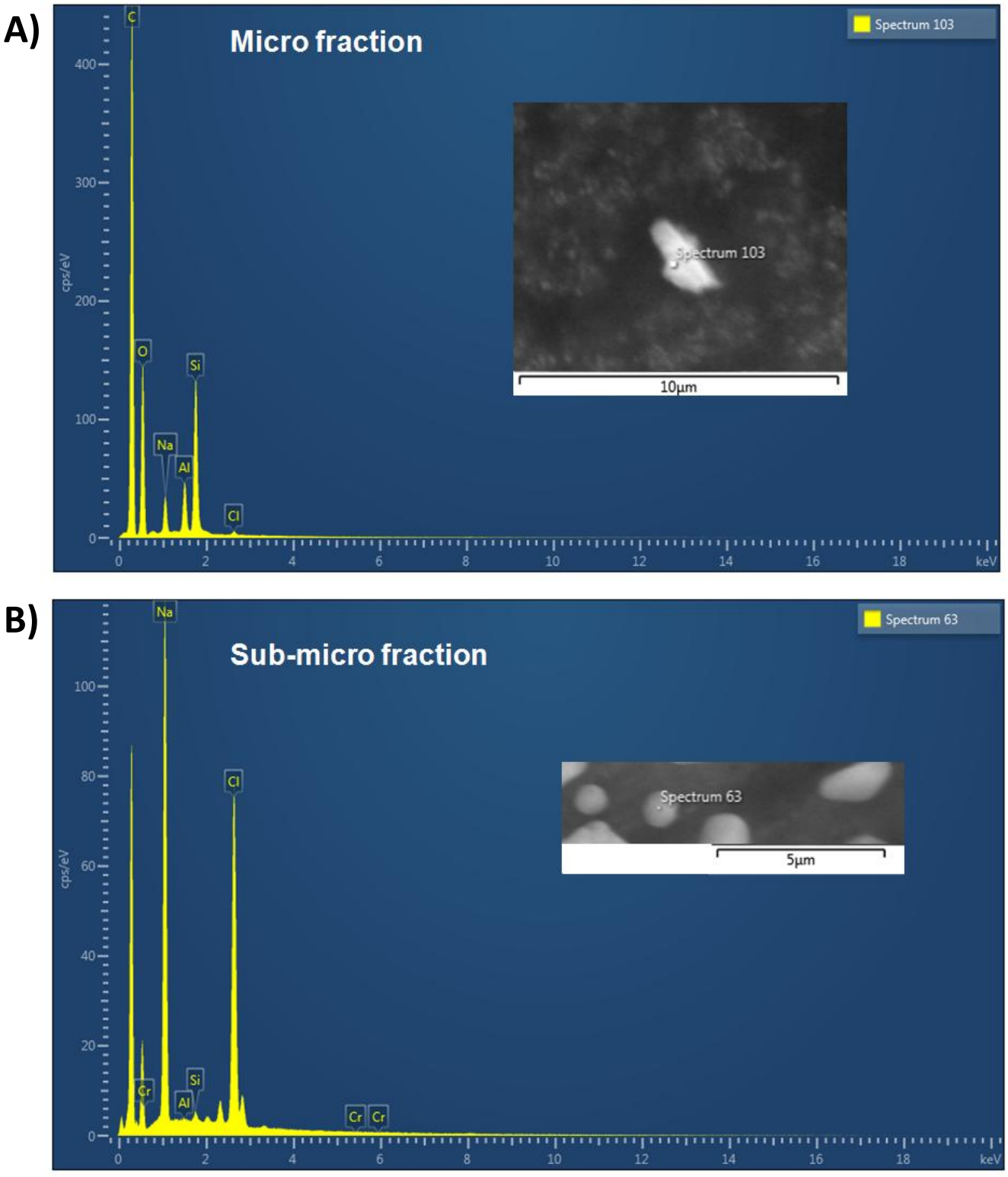

A)
Micro fraction
Spectrum 103
C
O
Si
Na
Al
Cl
cps/eV
keV
10µm
B)
Sub-micro fraction
Spectrum 63
Na
Cl
Si
Al
Cr
Cr
Cr
5µm

Figure 4

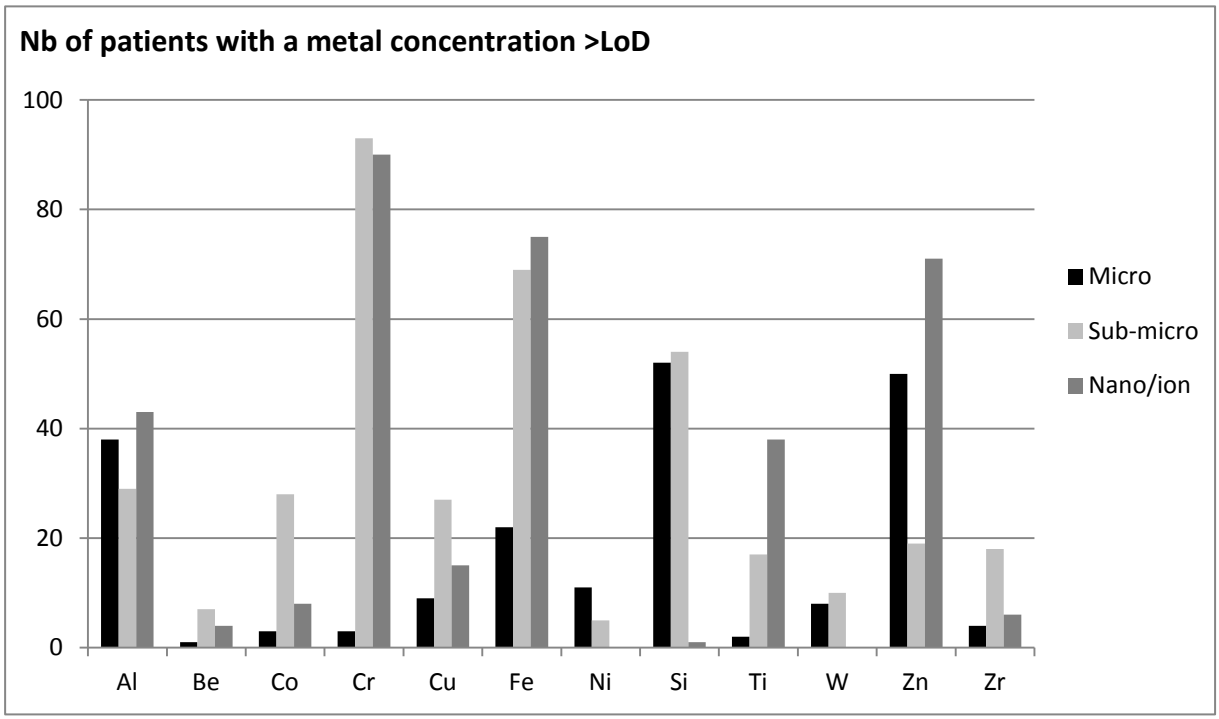
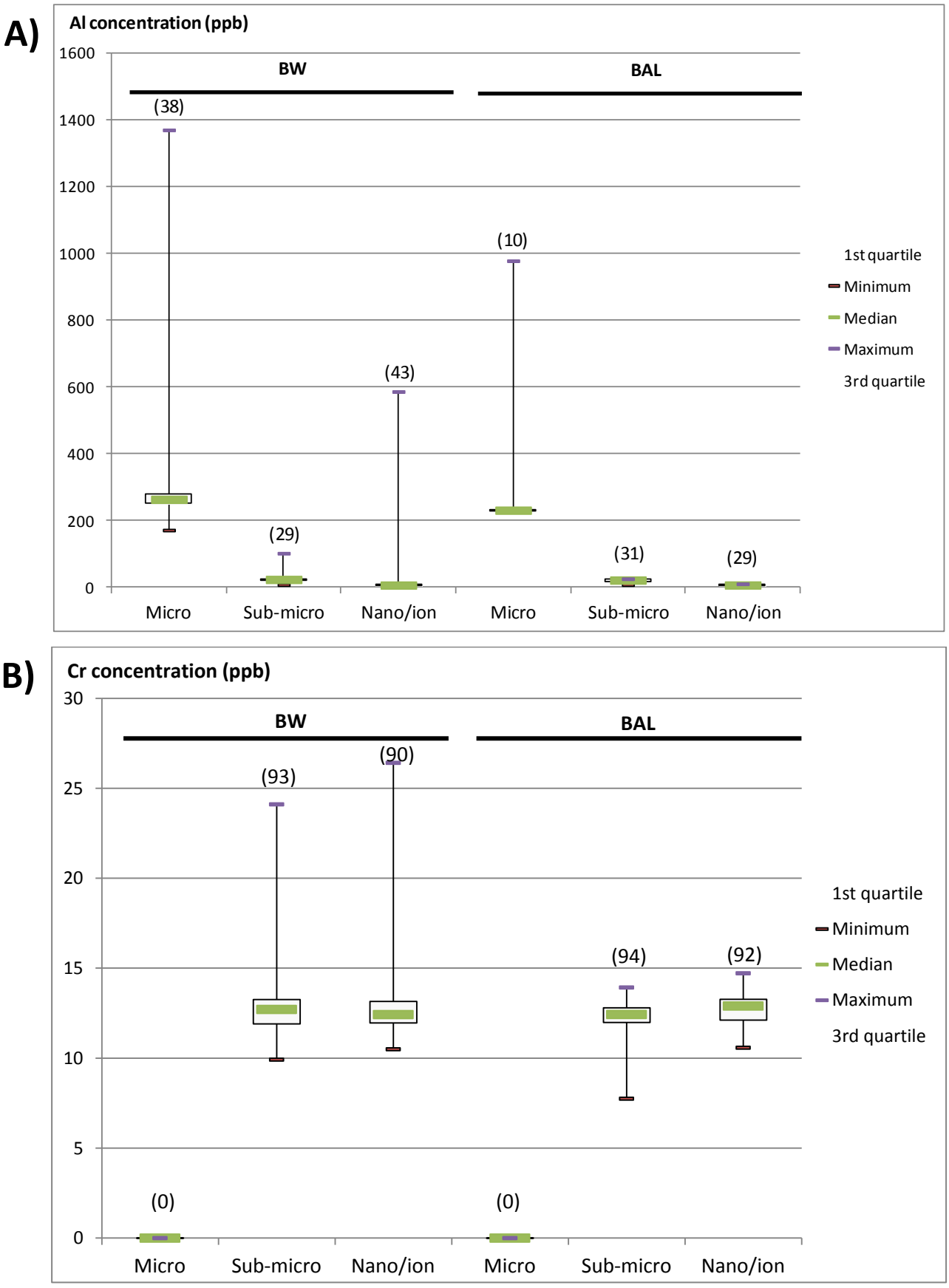

Nb of patients with a metal concentration >LoD
100
80
60
40
20
0
Al
Be
Co
Cr
Cu
Fe
Ni
Si
Ti
W
Zn
Zr
Micro
Sub-micro
Nano/ion

Figure 5


A)
Al concentration (ppb)
1600
1400
1200
1000
800
600
400
200
0
BW
BAL
(38)
(29)
(43)
(10)
(31)
(29)
Micro
Sub-micro
Nano/ion
Micro
Sub-micro
Nano/ion
1st quartile
Minimum
Median
Maximum
3rd quartile
B)
Cr concentration (ppb)
30
25
20
15
10
5
0
BW
BAL
(93)
(90)
(94)
(92)
(0)
(0)
Micro
Sub-micro
Nano/ion
Micro
Sub-micro
Nano/ion
1st quartile
Minimum
Median
Maximum
3rd quartile

C)

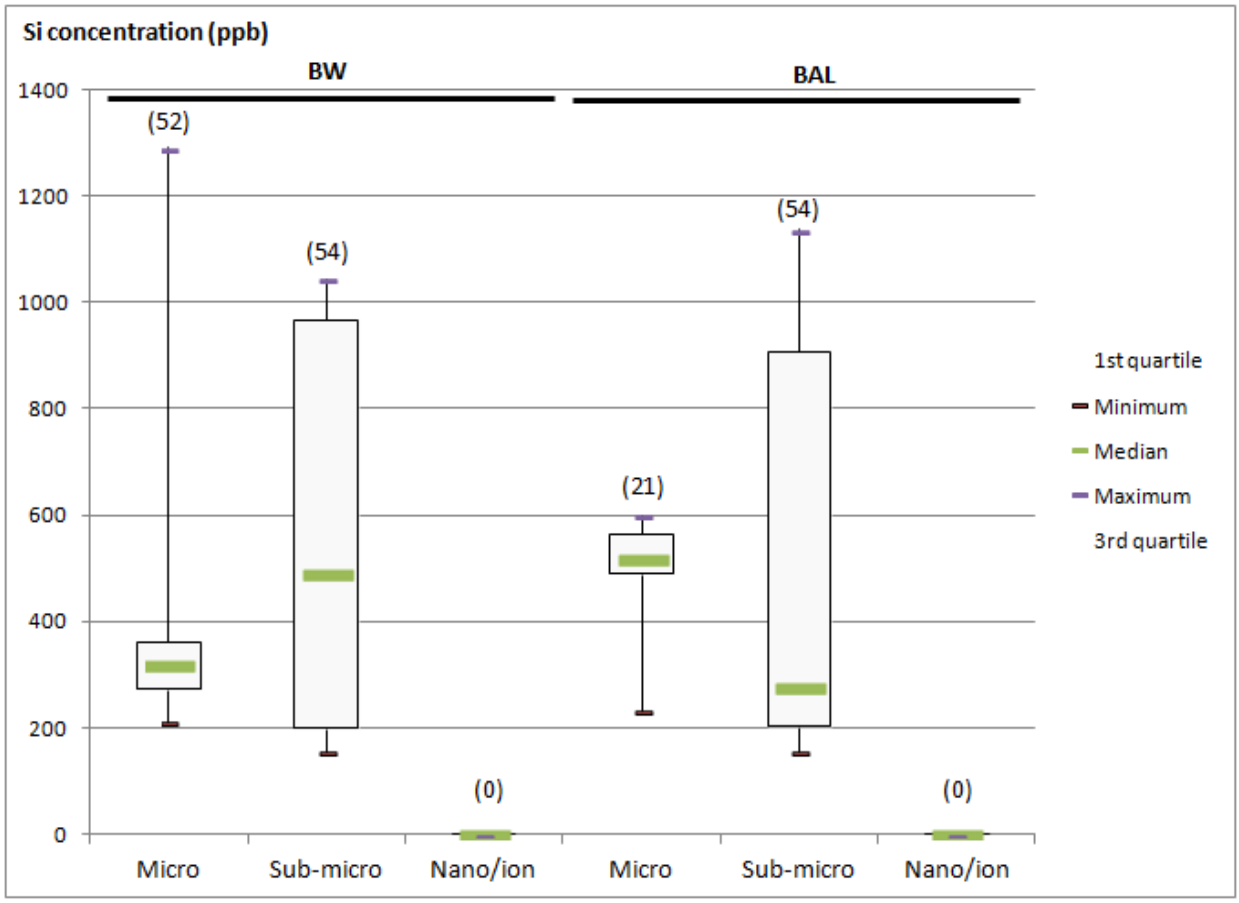

Si concentration (ppb)
BW
BAL
1400
1200
1000
800
600
400
200
0
(52)
(54)
(0)
(21)
(54)
(0)
Micro
Sub-micro
Nano/ion
Micro
Sub-micro
Nano/ion
1st quartile
Minimum
Median
Maximum
3rd quartile

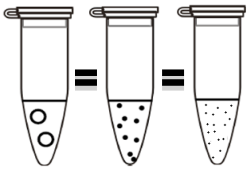
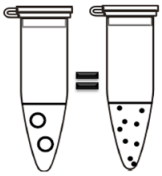
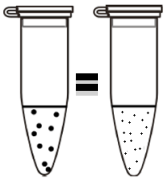
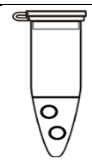
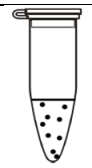
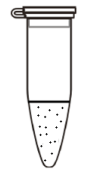
Table 1 – Global results for the ICP-AES analysis of the micro, sub-micro and nano/ion fractions of BW and BAL samples. A concentration of metals lower than the limit of detection (LoD) was considered as negative and was not included in the calculation of the mean. Therefore, mean concentrations of metals were calculated only considering patients with a value higher than the limit of detection (number indicated in the first column for each type of sample and fraction). Means and standard deviations (SD) are reported as well as the minimal (Min) and maximal (Max) concentration observed in each fraction.

	BW													
	Micro					Sub-micro					Nano/ion				
Metals	Nb of patients with a conc. > LoD	concentrations (ppb)				Nb of patients with a conc. > LoD	concentrations (ppb)				Nb of patients with a conc. > LoD	concentrations (ppb)			
		Mean	SD	Min	Max		Mean	SD	Min	Max		Mean	SD	Min	Max
Al	38	283	183	171	1370	29	31	23	7	102	43	67	145	5	586
Be	1	1	0	1	1	7	3	1	2	5	4	5	2	3	9
Co	3	11	8	5	23	28	2	2	1	7	8	4	2	1	7
Cr	3	3	0	3	3	93	13	3	10	24	90	13	2	10	26
Cu	9	31	7	22	44	27	80	127	15	395	15	26	6	15	32
Fe	22	136	172	65	894	69	29	73	9	615	75	95	228	10	1745
Ni	11	21	3	17	26	5	41	3	36	44	0	-	-	0	0
Si	52	387	216	207	1290	54	574	374	152	1043	1	812	0	812	812
Ti	2	120	118	2	238	17	5	3	3	13	38	10	17	2	68
W	8	33	14	14	56	10	86	91	23	312	0	-	-	0	0
Zn	50	14	9	7	55	19	94	127	6	330	71	15	11	6	45
Zr	4	18	24	3	59	18	12	7	3	26	6	29	34	6	102

	BAL													
	Micro					Sub-micro					Nano/ion				
Metals	Nb of patients with a conc. > LoD	concentrations (ppb)				Nb of patients with a conc .> LoD	concentrations (ppb)				Nb of patients with a conc. > LoD	concentrations (ppb)			
		Mean	SD	Min	Max		Mean	SD	Min	Max		Mean	SD	Min	Max
Al	10	310	223	227	978	31	19	5	7	25	29	7	1	5	10
Be	0	-	-	0	0	1	4	0	4	4	6	106	34	33	131
Co	0	-	-	0	0	22	1	0	1	2	3	1	0	1	2
Cr	2	4	1	4	5	94	12	1	8	14	92	13	1	11	15
Cu	21	33	12	21	65	13	19	3	15	23	1	16	0	16	16
Fe	31	117	89	66	441	55	21	24	9	131	49	37	101	10	562
Ni	0	-	-	0	0	0	-	-	0	0	0	-	-	0	0
Si	21	509	80	230	599	54	490	347	153	1136	0	-	-	0	0
Ti	1	19	0	19	19	5	3	0	3	3	24	2	0	1	3
W	29	17	4	13	29	0	-	-	0	0	7	265	303	21	855

Zn	90	12	4	7	29	15	13	6	6	22	15	9	2	6	12
Zr	0	-	-	0	0	10	8	10	3	36	4	34	41	9	104

Table 2 - Comparison of the metal content in the micro, sub-micro and nano/ion fractions of the BW and BAL in terms of number of patients exhibiting metal concentrations higher than the limit of detection. For this classification, a threshold was arbitrarily set at 10 patients.

		BW	BAL
Metals mainly found in the 3 fractions: micro, sub-micro and nano/ion		**Al** **Fe** **Zn**	**Al** **Fe** **Zn**
Metals mainly found both in the micro and sub-micro fractions		**Si**	Cu **Si**
Metals mainly found both in the sub-micro and nano/ion fractions		**Cr** Ti Cu	**Cr**
Metals found only in the micro fraction		Ni	W
Metals found only in the sub-micro fraction		**Co** **Zr** W	**Co** **Zr**
Metals found only in the nano/ion fraction		-	Ti

Supplementary data

Figure S1 – Number of patients with a metal concentration higher than the limit of detection (LoD) in BAL for the micro, sub-micro and nano/ion fractions.

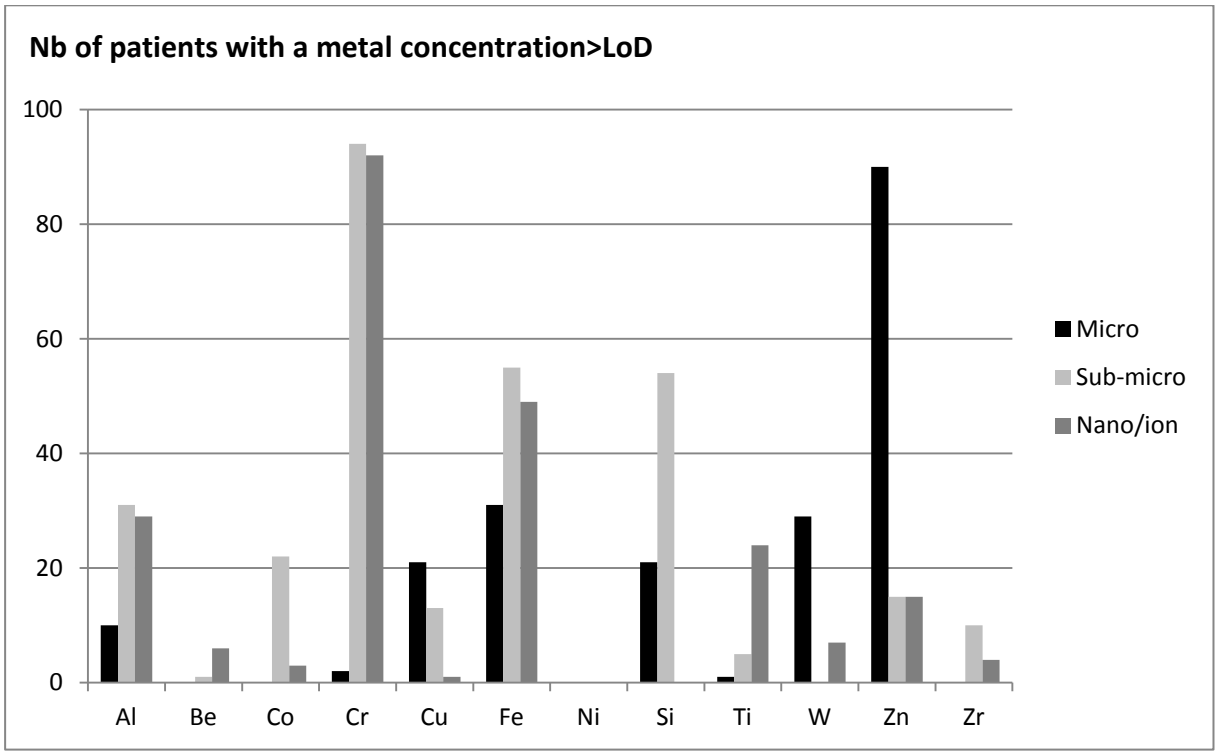
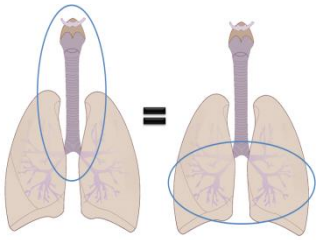
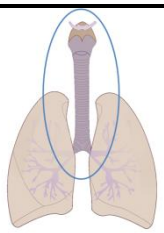
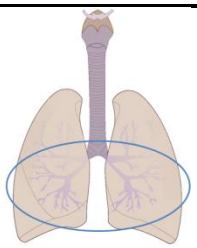


Table S1 – Comparison of the metal content between the BW and BAL regarding the micro, sub-micro and nano/ion fractions in terms of patients with a concentration higher than the limit of detection. For this classification, a threshold was arbitrarily set at 10 patients.

		Micro fraction	Sub-micro fraction	Nano/ion fraction
Metals similarly found in BW and BAL (>10 patients)		Al, Fe, Si, Zn	Al, Co, Cr, Cu, Fe, Si, Zn, Zr	Al, Cr, Fe, Ti, Zn
Metals similarly not found in BW and BAL (<10 patients)		Be, Co, Cr, Ti, Zr	Be, Ni	Be, Co, Ni, Si, W, Zr
Metals predominantly found in BW		Ni	Ti, W	Cu
Metals predominantly found in BAL		Cu, W	-	-

Statistical analyses patient by patient

1. Comparison of the metal load in BAL and BW:

The coefficients of correlation (R) were calculated following linear regression analyses and are reported in Table S2.

Table S2 – Calculation of the coefficients of correlation (R) between BAL and BW for each fraction and each metal. Correlations are indicated in bold.

	BAL vs BW		
	Micro fraction	**Sub-micro fraction**	**Nano/ion fraction**
Al	-0.1665	0.7413	0.5087
Co	N/A	0.0534	0.1204
Cr	-0.0392	0.1392	0.1260
Cu	-0.1140	-0.0425	-0.0453
Fe	0.2263	0.1757	0.2348
Si	0.3969	**0.9221**	N/A
Ti	-0.0114	0.0332	-0.0809
Zn	-0.0742	0.5730	0.7690
Zr	N/A	-0.0176	-0.0164

Despite an apparent similar metal load in BAL and BW, when comparing the metal concentration patient by patient for each fraction, no correlations appeared between BW and BAL except for Si in the sub-micro fraction (R=0.92).

2. Comparison of the metal load in the micro, sub-micro and nano/ion fractions:

The coefficients of correlation (R) were calculated following linear regression analyses and are reported in Table S3.

Table S3 – Calculation of coefficients of correlation (R) between the indicated fractions of BAL or BW for each metal. Correlations are indicated in bold.

	BAL			**BW**		
	Micro vs sub-micro fraction	**Micro vs nano/ion fraction**	**Sub-micro vs nano/ion fraction**	**Micro vs sub-micro fraction**	**Micro vs nano/ion fraction**	**Sub-micro vs nano/ion fraction**
Al	-0.1831	-0.0489	0.2843	0.3976	0.2397	**0.8658**
Co	N/A	N/A	0.3970	-0.0687	-0.0408	0.1518
Cr	0.0411	0.0630	0.5293	-0.0906	-0.1045	0.3731
Cu	-0.0389	0.1167	0.3015	-0.0514	-0.0678	0.6538
Fe	0.5918	0.4144	0.6193	0.1912	0.1985	**0.9133**
Si	**0.9368**	N/A	N/A	0.3805	0.0529	0.1642
Ti	-0.0245	-0.0603	0.3024	0.1099	-0.0065	0.6572
Zn	-0.0978	-0.1055	**0.8308**	0.0802	0.0834	0.6777
Zr	N/A	N/A	-0.0209	-0.0520	-0.0193	-0.0606

As shown by Table S3, with few exceptions (*i.e.* for Si in the micro and sub-micro fractions, Zn in the sub-micro and nano/ion fractions in BAL and for Fe and Al in sub-micro and nano/ion fractions in BW), no correlation was found between the metal load in the different fractions of BAL or BW, confirming the need to study each fraction independently.