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1 **Effects of occupational exposure to poorly soluble forms of beryllium on biomarkers of pulmonary**  
2 **response in exhaled breath of workers in machining industries**

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55 **ABSTRACT**

56 **Objective:** To analyze the effects of occupational exposure to poorly soluble forms of beryllium (Be)  
57 on biomarkers of pulmonary inflammation using exhaled breath condensate (EBC) in workers  
58 employed in machining industries.

59 **Methods:** Twenty machining operators were compared to 16 controls. The individual exposure to Be  
60 was assessed from the work history with several indices of exposure calculated on the basis of task-  
61 exposures matrices developed for each plant using historical air measurements. Clinical evaluation  
62 consisted in a medical questionnaire, measurements of biomarkers in EBC (tumor necrosis factor  
63 alpha (TNF- $\alpha$ ), total nitrogen oxides (NO $_x$ )), measurement of the fraction of exhaled nitric oxide  
64 (FeNO) and resting spirometry. Adjusted multiple linear regressions were used to study the effect of  
65 the exposure to Be on inflammatory biomarkers.

66 **Results:** Levels of TNF- $\alpha$  and NO $_x$  in EBC were not statistically different between exposed and  
67 controls. We found a statistically significant relationship between levels of TNF- $\alpha$  in EBC and both  
68 index of cumulative exposure and duration of exposure to Be. No other statistically significant  
69 relationships were found between exposure to Be and pulmonary response.

70 **Conclusion:** Our results suggest that machining-related exposure to Be is related to pulmonary  
71 inflammation involving TNF- $\alpha$ . These findings must be confirmed by larger studies.

72 **Keywords:** beryllium; exhaled breath condensate; tumor necrosis factor alpha; occupational  
73 exposure; machining.

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82 **HIGHLIGHTS**

- 83 • We studied the pulmonary inflammatory response to low exposure to beryllium (Be)
- 84 • TNF- $\alpha$  and nitrogen reactive species were assayed in exhaled breath condensate (EBC)
- 85 • Cumulative exposure to Be was correlated with TNF- $\alpha$  in EBC of machining operators
- 86 • No relationship between exposure to Be and nitrogen reactive species was found
- 87 • Low air exposure to Be is associated with pulmonary inflammation involving TNF- $\alpha$

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## 117 **1. INTRODUCTION**

118 Beryllium (Be) is a naturally occurring metal whose physical and mechanical properties are much  
119 sought-after in high technology industries, especially in the aeronautics, aerospace, energy,  
120 electrical, electronic and medical sectors. The Be industry produces 3 primary poorly soluble forms of  
121 Be: metallic Be, alloys of Be and oxides of Be. Exposure to Be and its compounds has been known to  
122 cause chronic beryllium disease (CBD), so called berylliosis, a long latency granulomatous disorder  
123 resembling sarcoidosis, that requires a prior Be sensitization phase (BeS) and primarily affects the  
124 lung (Balmes et al., 2014). The immunological reactions observed in affected individuals are Be-  
125 specific hypersensitivity responses involving CD4+ T-lymphocytes, production of T helper 1 (Th1) type  
126 cytokines and the subsequent generation of granulomatous inflammation in the lungs (Muller et al.,  
127 2011). Tumor necrosis factor-alpha (TNF- $\alpha$ ), a Th1-type cytokine, may exert effects on cell  
128 recruitment at site of inflammation and induce granulomatous structures (Semenzato et al., 2000).

129 Despite an increased understanding of the pathogenesis of BeS and CBD, little is known about  
130 relationships between external exposure, internal exposure and early respiratory effects. Once Be is  
131 deposited in a region of the respiratory tract, its chemical form and solubility are important exposure  
132 factors to pulmonary injuries (Stefaniak et al., 2004). Relatively insoluble Be compounds as well as  
133 copper-beryllium alloys were associated with development of CBD even in workers exposed at  
134 airborne concentrations of Be below the occupational exposure limit of 2  $\mu\text{g}\cdot\text{m}^{-3}$  (Schuler et al.,  
135 2005). Thus, poorly soluble species are thought to be responsible for local effects whereas soluble  
136 compounds are readily taken up and excreted with urine.

137 The use of a local matrix to monitor target tissue dose and effects occurring in the respiratory tract  
138 can be suggested when exposure to pneumotoxic substances occurs by inhalation (Mutti and  
139 Corradi, 2006). Exhaled breath condensate (EBC) represents one of these promising matrices. EBC  
140 collection is a completely noninvasive method for obtaining samples that reflect airway lining fluid  
141 composition through non-volatile substances (Kubáň and Foret, 2013). Biomarkers of local  
142 inflammation and oxidative stress - cytokines, nitric-oxide-related products, arachidonic acid  
143 metabolites, hydrogen peroxide, aldehydes - were reported as potential sensitive endpoints when  
144 identifying early biochemical changes in the airways of workers exposed to various pneumotoxic  
145 substances (Chérot-Kornobis et al., 2012). Nitric oxide (NO) is an important mediator involved in  
146 several physiological processes that induces respiratory tract injuries via the interaction of NO with  
147 reactive oxygen species resulting in reactive nitrogen intermediates (Sugiura et al., 2011; Robbins et  
148 al., 2000). NO-related products, which are found in EBC, are formed in the airways by oxidation of NO  
149 in nitrogen oxides (NO<sub>x</sub>, namely nitrite and nitrate) (Kubáň and Foret, 2013).

150 We recently reported that it was possible to quantify beryllium in the EBC of workers exposed mainly  
151 to water soluble forms of beryllium in a primary aluminum production plant (Hulo et al., 2016). We  
152 also shown that concentrations of Be in EBC and cumulative beryllium exposure index were  
153 significantly correlated.

154 To our knowledge, there have been no published data on exhaled biomarkers to assess respiratory  
155 tract effects in workers exposed to beryllium yet. Thus, the aim of the present study was to analyze  
156 the effects of occupational exposure to poorly soluble forms of Be on pulmonary inflammation using  
157 EBC in workers employed in machining industries.

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## 159 **2. MATERIALS AND METHODS**

### 160 *2.1. Study population*

161 We conducted an exposed-control study in a population of 20 workers exposed to poorly soluble  
162 forms of Be and 16 control subjects. Exposed subjects worked in three French plants (A, B and C) that  
163 machined:

- 164 - low beryllium alloys (copper – beryllium (Cu-Be) alloys with beryllium content 2% for plant A  
165 and 3% for plant B). Eight exposed workers were recruited from plants A and B
- 166 - high beryllium alloy with aluminum (Al-Be) (beryllium content 62%) and beryllium metal for  
167 plant C. Twelve exposed workers were recruited from plant C.

168 Machining operations within the plants consist mainly in lathe work, milling, grinding, deburring,  
169 filling, polishing, sanding and electrospark machining. Plants were identified from a French  
170 assessment survey of exposure associated with occupational use of Be and its compounds ((Vincent  
171 et al., 2009) and recruited on a voluntary basis of their management boards. Within each recruited  
172 plant, we identified workrooms and workstations with potential exposure to beryllium. All the  
173 operators working in these workrooms and workstations were proposed to participate to the study.  
174 The voluntary participants were informed about the protocol study and signed an informed and free  
175 consent form before enrollment.

176 Controls were enrolled at the same plants as the exposed subjects and were recruited on the  
177 statement of no current or previous Be exposure. We checked during onsite plant visits that controls'  
178 activities were disconnected from Be-exposed working areas and were performed in specific  
179 buildings far from Be-exposed working areas. Seven controls were recruited from plants A and B, and  
180 nine controls from plant C.

### 181 *2.2. Study protocol*

182 The study protocol was approved by the French national data protection commission and by the East  
183 ethics committee on protection of persons. All the subjects were recruited in 2010-2011 and  
184 answered a interviewed questionnaire in two parts:

185 - A medical questionnaire to assess medical history and treatment, in particular respiratory health  
186 and smoking habits. We used the questionnaire of the European Community for Steel and Coal  
187 on respiratory symptoms (Minette, 1989).

188 - A questionnaire to gather occupational history, focusing on the type, the duration and frequency  
189 of Be exposed operations as well as occupational exposure to other pneumotoxicants such as  
190 aluminum, titan, zirconium, welding fumes, copper, cobalt, antimony, iron, silica, asbestos, oil  
191 mist, nickel, chrome, refractory ceramic fibers, chlorine, solvents.

192 Then we collected the following samples in that order:

- 193 - collection of EBC samples
- 194 - measurement of fraction of exhaled nitric oxide ( $F_eNO$ )
- 195 - spirometric measurements

196 All clinical data were collected during the workshift.

### 197 *2.3. Assessment of exposure to beryllium*

198 Workers' exposure to Be was related to machining tasks performed on alloys Cu-Be 2-3% in plants A  
199 and B, and on alloy Al-Be 62% and Be metal in plant C. Beryllium exposed operations and tasks were  
200 recorded for each of the three plants based on information collected during the plants visits and  
201 workstation observations before the subjects' recruitment. For each Be-exposing task, exposure to  
202 Be was assessed using historical plant hygiene data issued from measurements campaigns that were  
203 performed in the 3 plants between 1998 and 2011. Using historical air measurements, task-exposure  
204 matrices were developed for each plant allowing the estimation of a level of exposure to Be for  
205 relevant operations.

206 Inhalable fraction of aerosols taken in personal and area samples were collected at a flow rate of 1  
207 or 2 L.min<sup>-1</sup> (GilAir™ pumps, Gilian Instrument Corp., West Caldwell, N.J.) using 37-mm diameter  
208 closed-face cassette (Merck Millipore Billerica, MA, USA) with a 4-mm diameter orifice containing a  
209 quartz fiber filter or a cellulose ester membrane (Merck Millipore Billerica, MA, USA). Once the  
210 sample had been taken, the internal walls of the cassettes were rinsed with 3 mL of nitric acid. Three  
211 milliliters of sulfuric acid and the rinse liquid was added to the membrane and subsequently  
212 mineralized by microwave. Volume was then adjusted to 25 mL with ultrapure HNO<sub>3</sub> 2%.  
213 According to French standards, analyses were performed either by ICP-MS following NF X 43-275  
214 before 2010 (NF X 43-275, 2002) or ISO 30011 after 2010 (ISO 30011, 2010) or by electrothermal  
215 atomic absorption spectrometry. Overall these air sampling protocols are validated to quantify  
216 exposure levels of beryllium above 1 ng.m<sup>-3</sup>.

217 Several indices of exposure were determined for each subject on the basis of the job histories:

- 218 - "total years" is the duration of beryllium exposed jobs defined as the total number of  
219 years of potential beryllium exposure (in years)



- 220 - “weighted Be exposure duration” (WBED), calculated by summing the products of the  
221 duration of each exposed task and the frequency of the task through all work history (in  
222 years) (Benke et al., 2000)
- 223 - “cumulative Be exposure index” (CBEI), which represents the lifetime sum of the  
224 products of the duration, frequency and beryllium exposure level for each beryllium  
225 exposed task (in  $\text{ng}\cdot\text{m}^{-3}\cdot\text{years}$ )
- 226 - “current Be exposure index” represents the sum of products of the beryllium level and  
227 frequency for each task performed at the time of interview (in  $\text{ng}\cdot\text{m}^{-3}$ )
- 228 - “alloy Be content” with 3 levels: controls, low Be content and high Be content. Exposed  
229 subjects from plants A and B were classified in “low Be content” (8 workers), exposed  
230 subjects from plant C were classified in “high Be content” (12 workers).

231 Subjects were also questioned on current and past co-exposure to known pneumotoxic species other  
232 than Be. Information on the number of pneumotoxic species, the duration and the frequency of  
233 associated operations in workplace were collected. Subsequently, a cumulative co-exposure index  
234 (CEI) was calculated for each subject as above.

#### 235 2.4. Assessment of pulmonary response

##### 236 *Collection of EBC*

237 The exhaled breath condensate (EBC) samples were collected at  $-4^{\circ}\text{C}$  using a Transportable Unit for  
238 Research on Biomarkers Obtained from Disposable Exhaled Condensate Collection Systems (TURBO-  
239 DECCS) device (ItalChill, Parma, Italy) according to the latest recommendations (Horvath et al., 2005).  
240 A disposable device consisting in a mouthpiece connected to a one-way aspiration valve, a saliva trap  
241 and a collection tube allowed to collect at least 2 ml of EBC during about 40 minutes of tidal  
242 breathing through the mouthpiece. Collected volumes of EBC (median(IQR)) were not statistically  
243 different (4.1 ml (2-5.5) in exposed group vs 4.25 ml (3.6-4.9) in control group).

244 All EBC samples were stored and transported at  $-20^{\circ}\text{C}$  and finally stored at  $-80^{\circ}\text{C}$ . All biomarkers  
245 measurements in EBC were performed altogether at the end of the recruitment.

##### 246 *Analysis of EBC*

247 The concentration of TNF- $\alpha$  was measured using an enzyme-linked immunosorbent assay (ELISA kit  
248 Human TNF- $\alpha$  Ultra sensible, Invitrogen, DO 450 nm). Our limit of detection was 0.09  $\text{pg}/\text{ml}$ . The  
249 average of the coefficient of variation for intra-assay variability was 9.5% (SD=8.5%).

250 The total nitrogen oxides ( $\text{NO}_x$ , namely nitrite and nitrate) were assayed by the Griess method  
251 (Griess Reagent Kit, Invitrogen, Cergy Pontoise, France) with spectrophotometric detection by two  
252 measurements: at first, nitrite was measured by the Griess reaction and then nitrate was reduced  
253 enzymatically to nitrite by nitrate reductase (Sigma, St. Quentin Fallavier, France). The concentration  
254 measured represents the sum of the nitrites and nitrates initially present in the EBC. Nitrate content

255 of EBC was calculated based on the difference between both measurements. Our limit of detection  
256 (LOD) was 2  $\mu\text{M}$ . Intra-assay variability expressed as mean (SD) of coefficients of variation was  
257 4.9% (4.7).

#### 258 *Fraction of exhaled nitric oxide ( $F_e\text{NO}$ ) measurement*

259 FeNO was measured by chemiluminescent detection of FeNO (ENDONO 8000<sup>®</sup>, SERES) according to  
260 the international recommendations (ATS/ERS Recommendations, 2005). Subjects were seated and  
261 nose clips were not used. After deep inspiration, NO concentrations were measured during  
262 controlled expiration, at the first stable  $F_e\text{NO}$  plateau for at least three seconds (FeNO variations <  
263 10% or 1 ppb).  $F_e\text{NO}$  was recorded as mean of two FeNO measurements at 50 mL/s expiratory flow.

#### 264 *Spirometric measurements*

265 Pulmonary function tests were performed according to the 2005 guidelines of the European  
266 Respiratory Society (Miller et al., 2005) using the portable SpiroDyn'R spirometer (Dyn'R society,  
267 Muret, France). Standard measures of pulmonary function were collected including forced vital  
268 capacity (FVC), forced expiratory volume in the first second ( $\text{FEV}_1$ ),  $\text{FEV}_1/\text{FVC}$  ratio, and forced  
269 expiratory flow at 25% and 75% of vital capacity (FEF25-75). Data were expressed as ratios of  
270 observed/predicted values using reference values of the Global Lung Function Initiative (Quanjer et  
271 al., 2012)

### 272 2.5. Statistical analysis

273 Descriptive statistics of exposure parameters as well as clinical and biological data were computed  
274 for both exposed and control groups. The log-transformed values were used for the FeNO analysis.  
275 The comparisons of means of quantitative parameters were performed using Student t-tests and the  
276 comparisons of qualitative parameters using Chi-square test. The correlation between two  
277 continuous parameters was assessed with Spearman's correlation coefficient.

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279 for both exposed and control groups. The log-transformed values were used for the FeNO analysis.  
280 The comparisons of means of quantitative parameters were performed using Student t-tests and the  
281 comparisons of qualitative parameters using Chi-square test. The correlation between two  
282 continuous parameters was assessed with Spearman's correlation coefficient.

283 Relationships between exposure to Be and health outcomes were analysed using multiple linear  
284 regressions. These outcomes were the log-transformed exhaled biomarkers (TNF- $\alpha$  and NO $_x$  in EBC,  
285 FeNO) and pulmonary function parameters. Independent variables included adjustment factors and  
286 exposure indices. Each index of exposure to beryllium was tested separately in different models. For  
287 quantitative indices the logarithm of the index (to which was added one so the transformed index is  
288 zero for unexposed workers) was used as independent variable. Both exposed workers and controls  
289 were included in the analyses.

290 The adjustment factors were selected based on their potential effects on airway inflammation.  
291 Multiple linear regressions were adjusted for history of pulmonary disease (presence of asthma,  
292 nasal polyposis, chronic obstructive pulmonary disease, emphysema, diffuse interstitial fibrosis),  
293 smoking status (smokers/ex-smokers/non-smokers), gender, age, and co-exposure index. We tested  
294 interactions between adjustment factors but they were not statistically significant.  
295 The statistical analyses were performed with STATA software (v. 14, Statacorp LP). A two-sided  
296 significance level of 0.05 was chosen for all tests.

### 297 **3. RESULTS**

#### 298 *3.1. General characteristics of the study population*

299 General characteristics are shown in **Table 1**. There was no significant difference in clinical data  
300 (respiratory history and symptoms) between controls and exposed subjects. There were more  
301 women in control group ( $p=0.01$ ). All spirometric indices were above the lower limit of normal and  
302 there were no statistically significant differences between exposed group and controls.

#### 303 *3.2. Assessment of beryllium exposure*

304 Exposure matrices were developed for each plant in order to assign levels of exposure to each task.  
305 Altogether, 36 specific job tasks were defined for individual exposure reconstruction as following:

- 306 - **Plant A** – eight tasks with exposure to alloy copper-beryllium 2%: lathe work, milling,  
307 sanding, electrospark machining, welding, polishing, grinding, manual filling
- 308 - **Plant B** – five tasks with exposure to alloy copper-beryllium 3%: deburring with a clipping  
309 tool, etching, wet machining, polishing, assembling
- 310 - **Plant C** – twenty-three tasks with exposure to beryllium metal and alloy aluminum-  
311 beryllium 62%, performed with different machines and belonging to the following  
312 operations: machining, dry machining, manual scraping, production support operations  
313 (maintenance, shipping and receiving, decontamination and cleaning operations,  
314 inventory management, waste package, supervision, quality control, machines  
315 assistance, workplace sampling and analysis)

316 Due to the confidentiality agreements with the companies, the results of beryllium exposure levels  
317 for each task cannot be provided here.

318 The highest Be concentration was measured in plant C on personal air samples collected in 1998 in  
319 Be machining workroom ( $3 \mu\text{g}\cdot\text{m}^{-3}$ ). All the other airborne concentrations of beryllium were below  
320 the current French occupational exposure limit value of  $2 \mu\text{g}\cdot\text{m}^{-3}$ . In contrast, a quarter of these  
321 concentrations were above the limit value of  $0.05 \mu\text{g}\cdot\text{m}^{-3}$  proposed by the American Conference of  
322 Governmental Industrial Hygienists (ACGIH, 2010).

323 The different indices of exposure were calculated for exposed subjects and controls and are  
324 presented according to alloy Be content (**Table 2**).

325 Occupational co-exposures reported by the workers were the following: copper, oil mist, iron, silica,  
326 chrome, welding fumes and solvents in plants A and B; aluminum, iron, titan, solvents and oil mist in  
327 plant C.

328 Cumulative co-exposure index was twofold higher in plants with low Be alloys exposure (plant A and  
329 plant B) than in the plant with high Be alloys exposure (plant C).

### 330 *3.3. Exhaled biomarkers*

331 The measurements of exhaled biomarkers in controls and exposed are presented in **Table 3**  
332 according to alloy Be content.

333 NO<sub>x</sub> and TNF- $\alpha$  were quantified in all samples of EBC. Levels of exhaled biomarkers of exposed  
334 subjects were slightly higher than those of controls but these differences were not statistically  
335 significantly different. Measurements of NO<sub>x</sub> in EBC and FeNO were not statistically significantly  
336 correlated (Spearman's correlation coefficient was 0.05 ( $p=0.79$ )). Also, EBC biomarkers NO<sub>x</sub> and  
337 TNF- $\alpha$  were not correlated (Spearman's correlation coefficient was -0.25 ( $p=0.14$ )) as well as TNF-  $\alpha$   
338 in EBC and FeNO (Spearman's correlation coefficient was 0.07 ( $p=0.67$ )).

339 EBC biomarkers TNF- $\alpha$  and NO<sub>x</sub> were not correlated with any of spirometric parameters (data not  
340 shown).

### 341 *3.4. Relationships between cumulative exposure and exhaled biomarkers*

342 The results of the multiple linear regressions are shown in **Table 4**.

343 The adjusted models showed that higher are the weighted Be exposure duration (WBED) or the  
344 cumulative Be exposure index (CBEI), higher are the TNF- $\alpha$  levels in EBC. The corresponding  
345 coefficients of determination for these models are 0.19 and 0.20, respectively. These results are  
346 unchanged when adjusting on pack-years during lifetime instead of smoking status (data not shown).  
347 Neither NO<sub>x</sub> in EBC nor FeNO levels were statistically significantly related to indices of beryllium  
348 exposure.

349 Likewise, spirometric measurements were not statistically significantly related to parameters of  
350 exposure to Be (data not shown).

351

## 352 **4. DISCUSSION**

353 To our knowledge, this pilot study is the first that analyzes exhaled biomarkers in active workers  
354 exposed to low atmospheric concentration of different poorly soluble Be species. Firstly, we found  
355 that the levels of NO<sub>x</sub> and TNF- $\alpha$  in EBC of subjects currently exposed were slightly higher than those  
356 of controls but not statistically different. Secondly, we have shown a statistically significant  
357 relationship between cumulative exposure to beryllium and the level of TNF- $\alpha$  in EBC. Finally, we  
358 have not found relationships between exposure to beryllium and nitrogen reactive species, for both  
359 volatile (FeNO) and nonvolatile (NO<sub>x</sub> in EBC) nitrogen intermediates.

360 All exposed subjects of our study were employed in machining industries and were exposed to poorly  
361 soluble forms of beryllium. Machining beryllium releases a large amount of particles of small size -  
362 namely more than 50% of the particles in the breathing zone < 10 µm size and 30% < 0.6 µm - that  
363 are likely to result in increased lung deposition compared to greater particles (Martyiny et al., 2000).  
364 Hoover et al (1990) has reported that milling processes, compared to sawing, produces Be particles  
365 with a smaller mass median aerodynamic diameter and a greater proportion of particles < 5µm in  
366 diameter. Very small (i.e., ≤2.5 µm) particles penetrate to the deep lung and are associated with  
367 increased risk of CBD. Respirable size particles with poorly soluble Be that deposit in alveolar region  
368 of the lung are rapidly engulfed by macrophages where they undergo dissolution and slow clearance,  
369 resulting in longer Be retention, greater opportunity for interaction with the immune system and  
370 formation of granulomas and chronic inflammation, which is characteristic of CBD (Stefaniak et al.,  
371 2006). The insoluble molecules in the epithelial lining fluid have an alveolar residence time that is  
372 potentially very long, unlike water-soluble particles that can be phagocytized or cross the alveolar  
373 epithelium through transcytosis (Oberdörster, G. et al., 2007).

374 The historical Be air measurements used in our study were rather low, and, consequently, the values  
375 of calculated indices of exposure. Most of air Be concentrations were below the limit value of  
376 0.05 µg.m<sup>-3</sup> proposed by the ACGIH and all but one were below the current French occupational  
377 exposure limit value of 2 µg.m<sup>-3</sup>. These levels are consistent with those reported in French industry  
378 (Vincent et al., 2009).

379 Individual exposure were then assessed using task-exposure matrices specific to each plant based on  
380 historical air samples measurements of similar tasks. By summing the cumulative exposures of a  
381 worker over all tasks worked within a job title, the task-exposure matrix was shown to address the  
382 variability of exposure within the job title and reduce possible exposure misclassification using a job-  
383 exposure matrix (Benke et al., 2000). However the assessment of Beryllium exposure levels is  
384 affected by the relative scarcity of available historical data; this may reduce the ability to detect a  
385 relationship between the estimated cumulative levels and the biomarkers of pulmonary response.

386 Other types of exposure parameters were calculated for each subject, some of which frequently  
387 reported such as “total years” (Harper et al., 2014), others calculated *ad-hoc* such as “current Be  
388 exposure index” or “weighted duration” of exposure to Be. These exposure parameters may be more  
389 robust than the cumulative exposure as they are not based on the task-exposure matrices.

390 Furthermore, they are less subject to the recall bias common in the retrospective assessment of the  
391 occupational exposure. Lastly, differences in Be content and alloys between plants A, B and C were  
392 taken in account in our study by regrouping plants and their workers according to the alloy Be  
393 content.

394 In the present study, the spirometric indices were adjusted for age, size and sex by recent GLI  
395 equations 2012 (Quanjer et al., 2012). Indeed, a recent study demonstrated that the equations GLI  
396 2012 provided a better fit in a French population, particularly among women (Hulo et al., 2016). We  
397 did not find significant difference in pulmonary function indices between exposed and controls. None  
398 of the exposed subjects showed an obstructive ventilatory disorder or decrease in vital capacity that  
399 could be suspected restrictive ventilatory disorder. These results can be partly explained by the low  
400 occupational exposure of the exposed group.

401 In contrast to spirometric or imaging testing which often indicate late and irreversible lung  
402 pathological process related to pneumotoxic species in workplace, the study of inflammatory and  
403 oxidative biomarkers in EBC which are likely to reflect the composition of the airway-lining fluid may  
404 be useful to assess early lung pathobiology accompanying or preceding inflammation (Mutti and  
405 Corradi, 2006).

406 TNF- $\alpha$  is a cytokine known to mediate and cause inflammatory reactions. It is noteworthy that the  
407 development of granulomatous inflammation in the lungs of patients with chronic beryllium disease  
408 (CBD) is associated with activation and accumulation of CD4+ T cells (Fontenot et al., 2002). T cells  
409 are activated in the presence of Be antigen and release proinflammatory Th1-type cytokines, mostly  
410 TNF- $\alpha$ , interferon-gamma and IL-2. Experimental studies of immunotoxicity following inhalation  
411 exposure in mice showed different pattern of cytokine secretion related to the chemical form of  
412 beryllium (Müller et al., 2011). Overall, in this latter study, inhalation of Be metal and Be oxide  
413 produces greater pulmonary toxicity than other forms. Although workers of our study were exposed  
414 to a low Be concentration, we have shown a relationship between duration and cumulative exposure  
415 to poorly soluble Be compounds and the level of TNF- $\alpha$  in EBC. In other words, being occupationally  
416 exposed on a full time basis during 10 years to an airborne Be concentration of 0.05  $\mu\text{g}\cdot\text{m}^{-3}$  (the  
417 occupational exposure limit proposed by ACGIH in 2010) is associated to an increase of 75% in TNF- $\alpha$   
418 in EBC. However, this result may be interpreted cautiously and, at the present time, we cannot  
419 speculate on the clinical relevance of this result. Our data suggest that TNF- $\alpha$  measured in EBC could  
420 mediate a nonspecific early step inflammation in workers exposed to low levels of Be by inhalation.  
421 As note, in the granulomatous disorder sarcoidosis resembling berylliosis, the release of Th1  
422 cytokines represents the earliest step in the series of events that lead to formation of pulmonary  
423 granulomas (Semenzato et al., 2000). Whether this inflammatory status could predispose to a  
424 progression to sensitization or berylliosis could not be addressed in this pilot study.

425 In our study, TNF- $\alpha$  was detectable in the EBC of all subjects, as in previous studies in chronic  
426 obstructive pulmonary disease (COPD) (Ko et al., 2009) but unlike in cleaning workers with asthma  
427 for which the detection rate was lower (30%) (Viczya et al., 2013). Values measured in our study  
428 were similar to those measured in other healthy population but were lower than those reported in

429 sarcoidosis (3.37 pg.mL<sup>-1</sup>) and in systemic sclerosis patients (13.5 pg.mL<sup>-1</sup>) (Mohan et al., 2016; Edmé  
430 et al., 2008). As salivary contamination could occur in EBC sampling, it is worth noting that salivary  
431 influence on the cytokine assessment in EBC may be negligible (Ichikawa et al., 2007).

432 Although FeNO is currently the most advanced airway inflammation marker in term of robustness,  
433 reproducibility and standardization of measurement (Kubáň and Foret, 2013), the nitrosative and  
434 oxidative stress assessed by non-volatile NOx exhibits satisfactory reproducibility and consistent  
435 stability and was largely reported in occupational settings (Chérot-Kornobis et al., 2012). Thus, nitrite  
436 concentration in EBC is significantly higher in workers exposed to chromium or welding fumes  
437 compared to controls (Murgia et al., 2006, Brand et al., 2010). We did not find correlation between  
438 the FeNO and NOx levels in our population, like a previous study which reported a lack of correlation  
439 in asthmatic patients (Chérot-Kornobis et al., 2011).

440 These results can be explained by the fact that NO could be metabolized in different products  
441 through multiple ways of the oxidative and nitrosative stress (Rahman et al., 2006, Chérot-Kornobis  
442 et al., 2011). We did not find any relation between the markers of oxidant or nitrosant stress  
443 measured in our study (NOx in EBC and FeNO) and the occupational exposure to beryllium.

#### 444 **Limitations**

445 Our study concerns a small number of subjects. Our recruitment forecasts were based on the study  
446 of Vincent et al. (Vincent et al., 2009) but recruitment of employees regularly exposed to beryllium  
447 was difficult as a consequence of the 2009 economic crisis. Moreover, industrial processes and  
448 specific regulations have evolved between the data published by Vincent and our recruitment  
449 campaign. In this study, workers were exposed to beryllium-containing alloys with different beryllium  
450 contents ranging from 2% to 100%, and this heterogeneity of exposure leads to a cautious  
451 interpretation of the results. Unfortunately, beryllium sensitization blood test was not performed  
452 and we did not dispose of BeLPT status or personal medical data. The limited availability of  
453 laboratories assessing BeLPT in France and medical data confidentiality were the main reasons.

#### 454 **CONCLUSION**

455 This pilot study reports several new findings relating biomarkers in EBC of workers in machining  
456 industries exposed to low levels of poorly soluble forms of Be. Taken together, the results suggest  
457 that machining-related exposure to beryllium seems to be associated to pulmonary inflammation  
458 involving TNF- $\alpha$ . Larger studies are necessary to confirm these results.

#### 459 **CONFLICT OF INTEREST**

460 The authors declare that they have no conflict of interest.

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