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PAPER

Does exposure to inflammatory particles modify the pattern of anion in exhaled breath condensate?

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Abstract

Exposure to environmental and occupational particulate matter (PM) induces health effects on the cardio-pulmonary system. In addition, associations between exposure to PM and metabolic syndromes like diabetes mellitus or obesity are now emerging in the literature. Collection of exhaled breath condensate (EBC) is an appealing non-invasive technique to sample pulmonary fluids. This hypothesis-generating study aims to (1) validate an ion chromatography method allowing the robust determination of different metabolism-related molecules (lactate, formate, acetate, propionate, butyrate, pyruvate, nitrite, nitrate) in EBC; (2) apply this method to EBC samples collected from workers exposed to quartz (a known inflammatory particle), to soapstone (a less inflammatory particle than quartz), as well as to controls. A multi-compound standard solution was used to determine the linearity range, detection limit, repeatability and bias from spiked EBC. The biological samples were injected without further treatment into an ion chromatograph with a conductivity detector. RTube® were used for field collection of EBC from 11 controls, 55 workers exposed to soapstone and 12 volunteers exposed to quartz dust. The analytical method used proved to be adequate for quantifying eight anions in EBC samples. Its sub-micromolar detection limits and repeatability, combined with a very simple sample preparation, allowed an easy and fast quantification of different glycolysis or nitrosative stress metabolites. Using multivariate discriminant analysis to maximize differences between groups, we observed a different pattern of anions with a higher formate/acetate ratio in the EBC samples for quartz exposed workers compared to the two other groups. We hypothesize that a modification of the metabolic signature could be induced by exposure to inflammatory particles like quartz and might be observed in the EBC via a change in the formate/acetate ratio.

Introduction

Increasing evidence indicates that exposure to environmental and occupational particulate matter (PM) induces health effects on the cardio-pulmonary system [1]. Indeed, particles with specific surface characteristics or chemical compositions can trigger signaling pathways through oxidative stress, inducing inflammation [2]. Beside this recognized effect, associations between exposure to PM and metabolic syndromes like diabetes mellitus [3–5] or obesity [6] are now emerging in the literature. In order to identify potential pathways associated with such effects, metabolomics techniques are often used. These techniques,

combining high-throughput mass spectrometry detection (MS) or nuclear magnetic resonance spectroscopy (NMR) with bioinformatics, allow the identification and quantification of small molecules resulting from biochemical processes taking place in cells, tissues or organs [7]. The final goal of such techniques is to find differences in the pattern of measured metabolites in order to differentiate groups.

As the lung is the main entry portal for the inhaled particles, it seems interesting to consider exhaled breath condensate (EBC) as a convenient way to access the airways lining fluid (ALF) and to determine in this liquid, a panel of biomarkers related to metabolic or oxidative stress effects [8]. Although this biological matrix is simply collected by cooling a subject's exhaled breath in a non-invasive way, there are inherent difficulties related to such a technique [9]. One of them is that analytical techniques need to be sensitive enough to detect selected biomarkers. Due to the very large water vapor content in the exhaled air, its condensation will strongly dilute the small fraction of the droplet originating from the ALF [10]. Sensitive, robust and validated analytical methods are thus a prerequisite to propose EBC as a routine clinical technique.

NMR spectroscopy appears to be a method of choice for multiple endogenous metabolites analysis in EBC as this technique is not destructive, very sensitive and reproducible [11–13]. Based on differences in the detected metabolites in this biological matrix, NMR technique could clearly differentiate healthy volunteers from patients with different pathologies like COPD or asthma [14, 15]. Among the metabolites often reported in EBC in such studies are short chain fatty acids (SCFA, like acetate, propionate, butyrate), α-keto acids (pyruvate,…) or formate. As all these organic acids are easily ionized in basic conditions, ion chromatography (IC) methods can be considered as a complementary alternative to the rather costly and sophisticated NMR technique. Reported levels of acetate, propionate, butyrate, formate, pyruvate and lactate as well as nitrite and nitrate in EBC by IC are still seldom [16–20].

The present hypothesis-generating study aims to (1) validate an IC method allowing the robust determination of metabolites from the glycolysis/tricarboxylic acid pathways (lactate, formate, acetate, propionate, butyrate, pyruvate) as well as metabolites, resulting from the nitrosative stress (nitrite, nitrate) in EBC, and (2) to apply this method to the EBC collected from two groups of workers exposed to quartz particles, a known inflammatory particle, and to soapstone particles, a less harmful particle, as well as to controls.

Materials and methods

EBC collection
Population
We conducted an exposed-control study on a population of 12 crystal quartz stone workers exposed to quartz dust, 55 workers exposed to soapstone dust, and 11 unexposed control subjects, all recruited between May and October 2014 in the state of Minas Gerais, Brazil. This population has been described in a former paper [21] and is briefly summarized here under.

The crystal quartz stone workers were producing decorative crystal objects in partially open work sheds. For that purpose, they had to cut, faceting, grinding, and polishing crystal quartz stones with the aid of motorized equipment and grinding or buffing wheels. In addition, silicon carbide dust or tripoli (silica flour) was used as loose abrasives for polishing. Such a population still presents a very high prevalence of silicosis and respiratory impairment [22]. The soapstone workers were producing ornamental objects and cooking tools like pans or pots. They shape soapstone blocks using saws or turning equipment combined with knives and sands. Most of the workers performed more than one task.

Controls not exposed to quartz or soapstone were recruited at the University of Ouro Preto (administrative sector and professors). All subjects were fully informed about the study's aims and gave their prior, free, and informed consent. The study was approved by the Ethics Committees on Research at the Federal University of Minas Gerais (Report 183/08) and the Federal University of Ouro Preto (reference 0063.0.238.000-10).

EBC collection
The EBC samples were collected during a single workday at a clean location away from the exposed subjects' workplaces. The EBC samples were collected over 15 min during tidal breathing using RTubes® (Respiratory Research Inc., Charlottesville, VA, USA) and nose clips, according to the latest recommendations [8]. As saliva contains a high concentration of different anions, subjects were asked to rinse their mouth with water before the collection and swallow the saliva during collection. We took care to avoid contamination and to isolate the EBC samples by sealing the used polypropylene tubes with caps, immediately after collection. The RTubes® containing the EBC were stored at $-80\,^{\circ}$C and subsequently thawed and aliquoted into Eppendorf tubes before transportation by plane to France.

Anion analysis and validation
Method description
Only polypropylene material/containers were used. Standard solutions were prepared in 0.2 μm filtered Milipore water (18.3 MΩ.cm, <3 ppb total organic carbon). Individual stock solution of each selected anion was prepared in polypropylene bottles by dissolving about 100 mg precisely weight of the salt (sodium form for lactate, acetate, propionate, butyrate, pyruvate, nitrite; ammonium salt for formate and

potassium salt for nitrate) in a known mass of water. The working standard solution containing the eight anions was prepared by adding known mass of each stock standard and diluting to the convenient mass with ultrapure water.

After thawing and vortexing the samples, 100 μl of the EBC samples were introduced in a low volume polypropylene vial for IC (0.3 ml, 11.5 × 32 mm screw vial, MachereyNagel Switzerland). Ten microlitres of this sample was then directly injected without additional treatment into a Dionex ICS 5000$^+$ ion chromatograph, equipped with a dual pump, eluent generator, autosampler and conductivity detector. The analytical column was a IonPac AS11-HC 250 mm, 4 μm (ThermoFisher Scientific, Ecublens, Switzerland), thermostated at 30 °C. The eluent gradient was 5 min at 1 mM hydroxyde ion than increasing to 35 mM during 5 min and finally staying at this concentration during 7 min.

The method's performance for each analyte was determined using the NFT 90–210 protocol [23]. The linearity of the calibration curve was evaluated in a range starting from sub-micromolar levels to about 3–15 times the levels measured in EBC. The limit of quantification (LOQ) was determined using an *a priori* estimation, and verifying that its accuracy was smaller than an acceptable maximal deviation from the LOQ, set at 60%. The limit of detection (LOD) then corresponds to one third of the LOQ. The method's accuracy and bias were evaluated by spiking a pooled EBC with three different concentrations (about 50, 100, 150% of the pooled EBC content). Five independent measurements were done at intermediate precision condition. Finally, we verified the possible contribution to the anion concentration from RTubes® by adding 2 ml of MiliQ water to each consumable, and shaking for at least 2 min. The stability of the EBC sample stored at −20 °C was determined by analyzing aliquots of a pooled EBC sample and a spiked EBC sample regularly over 12 months.

In order to get an idea of the inter-individual variations, we asked six different healthy volunteers to collect their EBC one time at three different respiratory rates (low, medium, and high respiratory rate at 8, 12, and 24 breaths.min^{-1}, respectively).

Statistical analysis

As the anion concentrations were not normally distributed, their log-normal transformation was considered in the statistical analysis. A first ANOVA analysis was done on these data in order to determine if differences between the three groups could be observed. A stepwise discriminant analysis was applied to determine the best subset of eight anions able to discriminate the three groups of subjects. The model parameter is Wilks' Lambda, an index of the discriminating power ranging between 0 and 1 (the lower the value, the higher the discriminating power). Then a

canonical discriminant analysis was performed to identify canonical axes, which are linear combinations of these selected anions that provide maximal separation between the subjects' groups. Statistical analyses were performed using SAS 9.4 software (SAS Institute Inc. Cary, USA) and Stata software (StataCorp LP, College Station, TX, USA).

Results

Analytical method validation

The selected analytical method was simple and allowed a fast determination of eight anions. Only about 100 μl of EBC was needed, allowing triplicate injections of 10 μl. All selected anions were detected and could be quantified in the different EBC samples. Typical concentrations were comprised between about 0.1 μM (pyruvate) to some 100 μM (acetate). The developed elution gradient (hydroxide) allowed a good separation of the different anions as illustrated in the figure 1.

Table 1 resumes the figure of merits for the developed analytical method. The calibration curve was linear for most of the analytes. Nevertheless, for acetate, propionate and nitrate, the use of a second-order polynomial curve fitting was selected as it increased the regression coefficient above r^2 = 0.99. The IC with conductivity detection was quite sensitive with limits of detection in the sub-micromolar concentration range. The bias observed at this concentration was indeed smaller than 60% of the LOQ, as proposed by the NFT 90–210 standard. The recovery rate obtained on spiked EBC samples were also very good (above 90% for all analytes). The averaged coefficient of variation associated with the measurement of spiked EBC at three levels in intermediate fidelity conditions was estimated for each analyte and are given in table 1. We could verify that the repeatability was smaller than 12% for all analytes, except for nitrate, which reached 20%. As certified material for the selected anions is not available, we estimated the analytical bias by using the spiked EBC samples. Following the NFT 90–210 standard, we verified that the normalized bias was smaller than 2, indicative of a negligible bias. The stability of the different anions in the EBC samples was tested by analyzing regularly aliquots of a pooled EBC sample, stored at −20 °C. The selected anions were stable at least 12 months at this temperature without any clear concentration change (within 20% of the average concentration) (figure S1 is available online at stacks.iop.org/JBR/14/026005/mmedia, supplementary material).

The presence of potential contaminations from the RTubes® was tested by washing the inner surface of three tubes with 2 ml of water. On average, concentrations of the different anions were below the limit of quantification, except for formate and nitrate. For these two anions, their contribution to typical EBC levels from Brazilian controls or exposed workers were
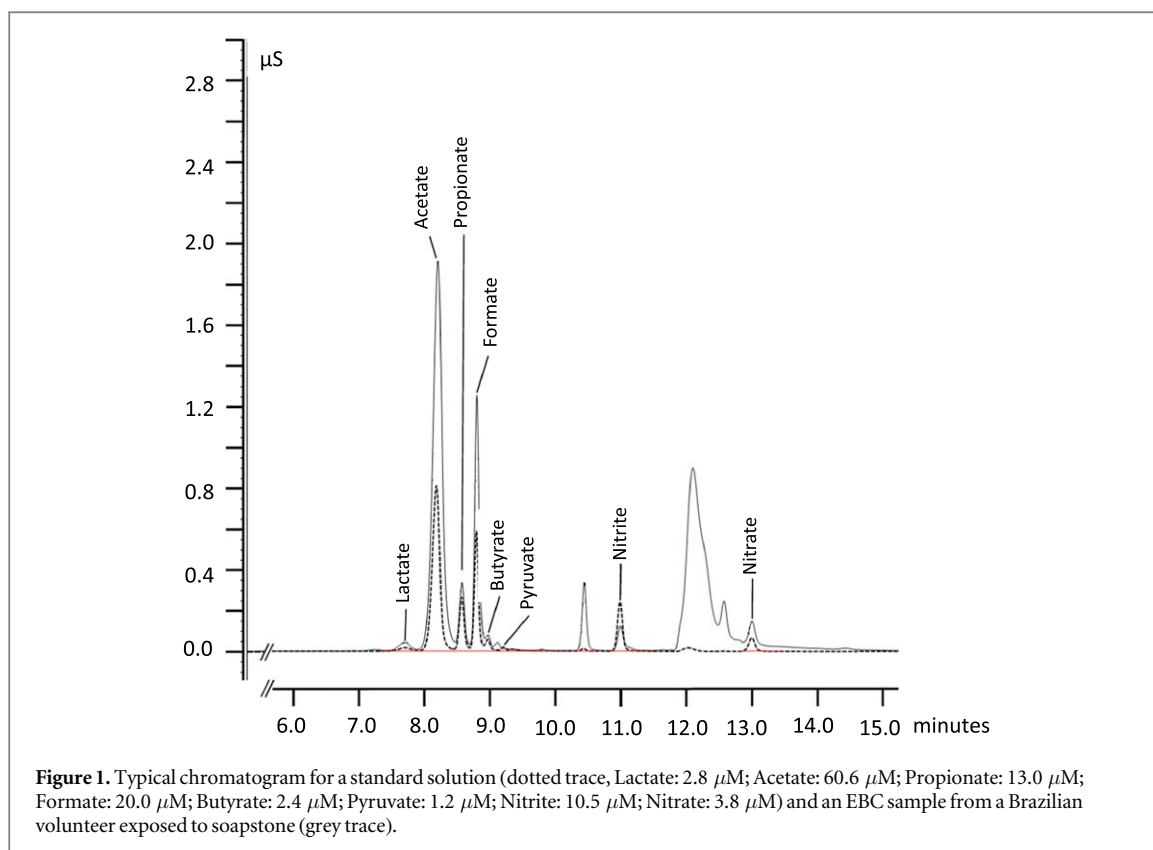
Figure 1. Typical chromatogram for a standard solution (dotted trace, Lactate: 2.8 μM; Acetate: 60.6 μM; Propionate: 13.0 μM; Formate: 20.0 μM; Butyrate: 2.4 μM; Pyruvate: 1.2 μM; Nitrite: 10.5 μM; Nitrate: 3.8 μM) and an EBC sample from a Brazilian volunteer exposed to soapstone (grey trace).

Table 1. Characteristics of the analytical method allowing the determination of eight anions in exhaled breath condensate.

	Lactate	Acetate	Propionate	Formate	Butyrate	Pyruvate	Nitrite	Nitrate
Calibration range [μM]	0.1–5.7	0.6–121	0.13–26	0.2–40	0.1–4.7	0.05–2.3	0.1–20.9	0.07–7.6
Regression type	Linear	Poly. X^{2a}	Poly. X^{2a}	Linear	Linear	Linear	Linear	Poly. X^{2a}
Limit of detection [μM]	0.11	0.27	0.07	0.12	0.58	0.16	0.20	0.07
Recovery EBC spiked $\approx$50% [%]	99 $\pm$ 4	99 $\pm$ 9	96 $\pm$ 2	99 $\pm$ 19	99 $\pm$ 13	101 $\pm$ 12	98 $\pm$ 6	97 $\pm$ 10
Recovery EBC spiked $\approx$100% [%]	101 $\pm$ 4	95 $\pm$ 6	92 $\pm$ 5	94 $\pm$ 11	96 $\pm$ 9	102 $\pm$ 9	96 $\pm$ 4	95 $\pm$ 2
Recovery EBC spiked $\approx$150% [%]	95 $\pm$ 4	92 $\pm$ 6	87 $\pm$ 7	90 $\pm$ 3	93 $\pm$ 6	93 $\pm$ 5	94 $\pm$ 6	90 $\pm$ 6
Averaged coefficient of variation [%]	7	8	9	12	9	7	8	20

[a] Second order polynomial regression

smaller than 5% for formate and 15% for nitrate (data not shown). Based on these results, the field collection of EBC was done with RTubes® without additional treatment as they are cleaned to factory standards (alcohol followed by deionized water and forced air-drying) before being packaged in plastic bags.
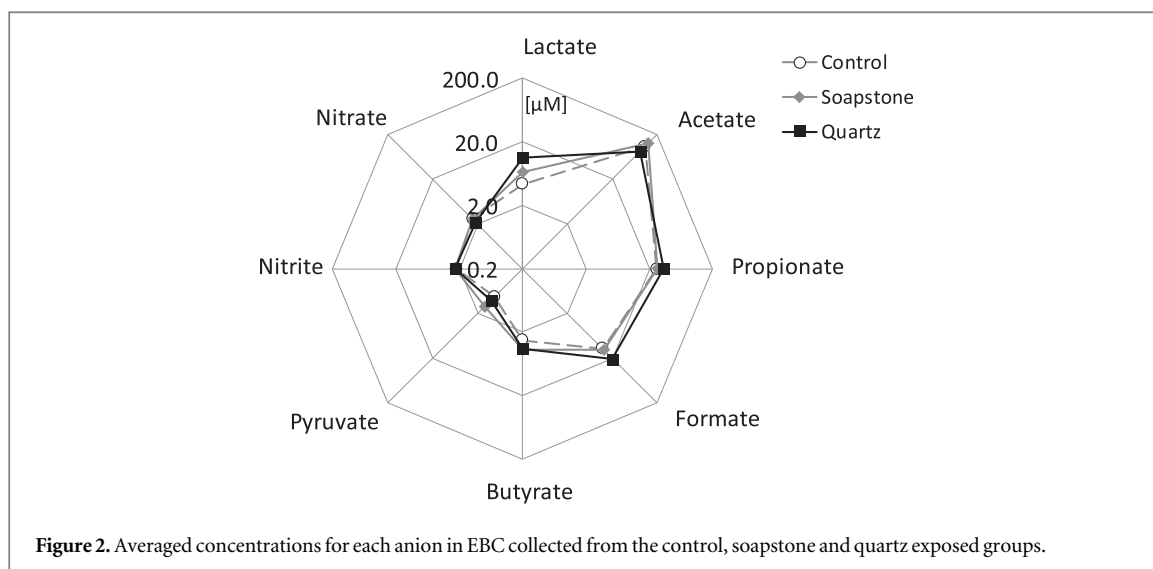
Inter-individual variability
The inter-individual variability for the selected anion concentration was determined by collecting at different days EBC samples from six healthy non-smoker volunteers not occupationally exposed to particles. For each volunteer, EBC collection was repeatedly done at three respiratory rates (low, medium, and high respiratory rate at 8, 12, and 24 breaths.min^{-1}, respectively).

The respiratory rate had no influence on the anion concentration (log transformed values, one-way ANOVA with Bonferroni multi-comparison correction, p > 0.05) except for lactate (table S1 supplementary material). For this anion, we observed a statistically significant increase in the EBC concentration for the highest respiratory rate compared to the other ones (p = 0.001). The inter-individual variability was rather large and comprised between 9% and 110%, depending of the analyte and the collection conditions (table S1 supplementary material).

Field study
Population and exposure assessment
The general characteristics of the studied population and the level of particulate exposure have been already published [21] (table S2, supplementary material).

Figure 2. Averaged concentrations for each anion in EBC collected from the control, soapstone and quartz exposed groups.

Briefly, the respirable dust concentration was the highest for the soapstone workers whereas the crystal quartz group had a higher silica dust exposure, as expected.

Concentration of anions in EBC

The selected anions could be quantified in all the EBC samples from either controls or workers exposed to soapstone or quartz. Figure 2 illustrates the averaged concentrations of each anions determined for each groups.

In this study, acetate presented the highest concentration for all measured anions followed by propionate > formate > lactate > butyrate > nitrite ∼ nitrate > pyruvate. This classification was the same for the three groups considered in this study. Acetate and propionate were highly correlated together (r^2 comprised between 0.82–0.87, depending on the exposure group) as well as propionate and butyrate (r^2 comprised between 0.62–0.86, depending on the exposure group). The correlation between acetate and butyrate was lower, with r^2 values comprised between 0.42 (soapstone group) to 0.76 (control group) (figure S2, supplementary material). The ratio of acetate: propionate:butyrate was 81 ± 6: 16 ± 6: 2 ± 1 for controls, 81 ± 5: 16 ± 4: 3 ± 2 for soapstone exposed workers and 75 ± 12: 21 ± 11: 5 ± 6 for quartz exposed workers. Acetate (log transformed concentrations) was the only variable presenting a statistically significant difference between groups, with a smaller level for the quartz group (one-way ANOVA with Bonferroni multi-comparison correction, $p = 0.01$) compared to the soapstone group.

A stepwise linear discriminant analysis was done using the eight selected metabolites and resulted in the identification of five anions able to discriminate the three subjects' groups: acetate, propionate, formate, nitrate and pyruvate (Wilks' Lambda = 0.64 $p < 0.001$). With these five anions, two canonical axes could be defined, with only one (Can 1) containing 93% of the information. The model for this Can 1 axis was:

$$\text{Can } 1 = 4.40 * \log[\text{acetate}] + 3.11 * \log[\text{nitrate}] + 2.25 * \log[\text{pyruvate}] - 5.34 * \log \times [\text{formate}] - 3.09 * \log[\text{propionate}]$$
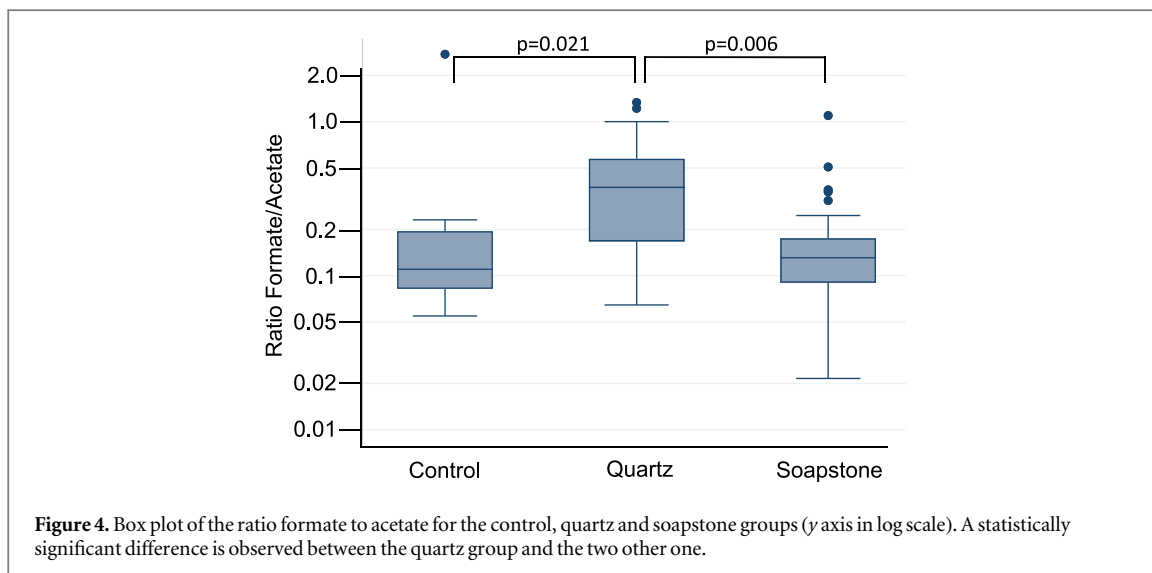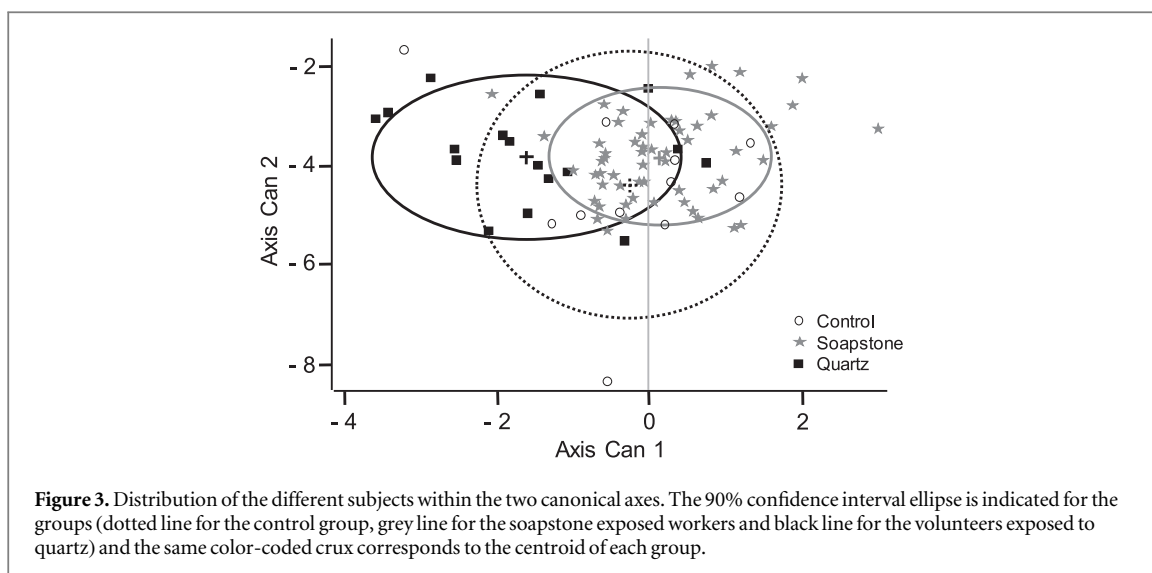
This model maximizes the difference between the two groups of exposed workers to soapstone and quartz, respectively, as illustrated in figure 3.

Two families of anions contribute differently to this model: acetate, nitrate and pyruvate contribute in a positive way whereas formate and propionate decrease the value of Can 1 axis. Along this axis (figure 3), the centroid of the ellipse corresponding to the 90% confidence interval for individuals exposed to soapstone is located slightly in the positive side whereas for individuals exposed to quartz, this centroid is located in the negative side of this canonical axe. Based on the model for Can 1, such observation suggests that either the negative contributing variables (formate and/or propionate) have a larger influence for quartz-exposed volunteers than for soapstone-exposed workers or that the contribution of positive variables (acetate, pyruvate, nitrate) is decreasing for the quartz group compared to the soapstone group.

By using the ratio formate/acetate as a measure of the contribution of these positive and negative families, we observed a statistically significant difference between quartz-exposed workers compared to the soapstone-exposed group as well as with the control (one-way ANOVA with Bonferroni correction, $p = 0.006$ and 0.021, respectively, figure 4).

Discussion

By using a validated ion chromatography method and a statistical treatment based on multiple linear discriminant analysis, we observed a different pattern of anions in EBC samples for quartz exposed workers

Figure 3. Distribution of the different subjects within the two canonical axes. The 90% confidence interval ellipse is indicated for the groups (dotted line for the control group, grey line for the soapstone exposed workers and black line for the volunteers exposed to quartz) and the same color-coded crux corresponds to the centroid of each group.

Figure 4. Box plot of the ratio formate to acetate for the control, quartz and soapstone groups (*y* axis in log scale). A statistically significant difference is observed between the quartz group and the two other one.

compared to the soapstone or control groups, with a lower formate to acetate ratio for these two last groups.

The analytical method used proved to be adequate for quantifying eight anions in EBC samples. Its sub-micromolar detection limits and repeatability, combined with a very simple sample preparation, allowed an easy and fast quantification of different glycolysis or nitrosative stress metabolites. The fact that the sample is directly injected without any treatment is an advantage as drying/concentrating steps in the EBC samples treatment are reported to induce solute precipitation, potentially creating insoluble species whose analysis is problematic [24].

Short chain fatty acids (SCFA) are the main metabolites resulting from anaerobic bacterial fermentation [25] or can originate from different pyruvate metabolism pathways [26]. At the cellular level, SCFA can be used as an energy source and are playing a role in the modulation of cell proliferation/differentiation as activation of immune/inflammatory responses [25, 27]. Our data indicates that concentrations of acetate, propionate and butyrate (in descending order of abundance) in EBC are the highest among the selected anions, in agreement with results from other literature (table S3 supplementary material). The strong correlation between acetate, propionate and butyrate suggest that these three anions are originating from the same process (figure S2 supplementary material). For EBC samples from healthy volunteers, the averaged physiological non de-aerated pH is calculated to be 7.2 ± 0.7 [28]. At such pH, acetate, propionate, butyrate and formate are all under the deprotonated form and are thus non-volatile (figure S3, supplementary material). Among the eight metabolites measured in this study, only acetate levels was significantly decreased for the quartz group compared to the control or soapstone group ($83 \pm 113 \, \mu M$ versus $105 \pm 90 \, \mu M$ and $127 \pm 135 \, \mu M$, respectively). Such observation is in line with *in vitro* studies. In a metabolomic study exposing HeLa cells to SiO_2 nanoparticles [29], an initial increase of metabolic end-products (including acetate) followed by a decreased release of

such compounds in the cell milieu was observed either for low or high SiO_2 dosage levels. This metabolic response was attributed to an initial high glycolytic activity followed by an adaptive response to silica nanoparticle exposure, characterized by a decreased energy demand of the cells due to apoptosis and cell death. A similar suppressive effect on acetate levels has been observed for hepatic cells (HepG2) exposed to silica nanoparticles [30]. Modifications of acetate levels with contrasting tendencies are also reported in studies aiming to compare metabolites fingerprints in bio-fluids collected from healthy volunteers or subjects with different pathologies [27]. Specifically for EBC matrices, acetate levels are reported to be lower for obese compared to lean subjects [31]. Such decrease has been linked to energy balance. In a similar study comparing metabolic EBC fingerprints of obese asthmatics (OA), lean asthmatics (LA) and obese non asthmatics (ONA) [32], a reduced level of acetate was determined for both OA and LA compared to ONA. On the contrary, EBC of COPD smokers presented an increased acetate level compared to healthy smokers [33].

We have shown, on a small number of resting subjects, that lactate concentration in EBC increases with respiratory rate. Marek *et al* [34] have shown in healthy subjects that the concentration of lactate increased two-fold in the EBC after a maximal exercise and that this was correlated with blood lactate concentration. In the condition of maximal exercise, lactate mainly comes from the anaerobic metabolism or results from a switch from oxidative phosphorylation (taking place into the mitochondria), to aerobic glycolysis (in the cellular cytosol) [35]. At rest, the lung production of lactate is equal to the lactate utilization [36], so in normal conditions without respiratory diseases, the lactate concentration assayed in the EBC corresponds to the lactate molecules coming from the pulmonary capillaries. Hyperventilation at rest for healthy subjects causes blood alkalosis due to excessive expiration of CO_2 and Druml *et al* [37] showed that respiratory alkalosis increases the basal plasma concentration of lactate. That's why we found higher concentrations of lactate in EBC for the higher respiratory rates. However, we found lower levels of lactate concentration in EBC of non-exposed subjects than other adults (table S3 supplementary material). This lower concentration could be influenced by the EBC collected device or by the dosing method.

Compared to the very few studies reporting formate concentrations in EBC [18, 19], the level measured within this study are about two times larger (table S3 supplementary material). Formate acts as a dual redox species as it can play an important role in the progression of inflammatory diseases [31]. On the other hand, formate results when glyoxylate reacts with ROS [38] and this carboxylic acid can be further used as a reducing compound to generate NADPH and succinate [39].

Pyruvate levels are the lowest in all the EBC samples, in accordance with data from Greenwald *et al* [18]. Pyruvate is an intermediate compound in the metabolism of carbohydrates (glycolysis), proteins and fats. In the presence of ROS, it is decarboxylated and generates acetate [38].

Averaged levels of EBC nitrite and nitrate are of similar magnitude for all groups in this study (figure 2), whereas generally, a larger concentration of nitrate is determined in healthy patients (table S3 supplementary material). Nitrite and nitrate have been reported to be present in the airway lining fluid and correspond to the end-product of NO metabolism [8]. These two anions are considered as good biomarkers of oxidative/nitrosative stress whereas their correlation with exhaled NO is low [40]. In our case, the nitrite concentration has to be considered with caution and could be overestimated due to the presence of an unidentified compound, which could not be completely resolved with the IC elution gradient. The largest variability was determined for nitrate (table 1). In a large population study [40], the intra-individual variability for nitrite and nitrate was 22% and considered as satisfactory. In another study [41], a similar intra-individual CV for nitrite concentration in healthy volunteers was reported whereas that of nitrate was larger, around 49%. The same study reported an inter-individual variability for nitrite and nitrate as high as 46% and 119%, respectively. Such a variability is difficult to attribute definitely to one reason but the presence of high levels of nitrite (max. 1–2 mM) and nitrate (max. 10 mM) in the oral cavity after dietary intake [42] could influence the EBC concentration levels.

The use of a linear discriminant analysis on these data allowed the development of a model maximizing the separation between quartz and soapstone groups. Acetate, nitrate and pyruvate concentrations positively influenced this separation, whereas formate and propionate presented a negative contribution. In order to avoid the fact that differences in concentration observed between groups are due to different dilution of the EBC samples, the use of a ratio of two non-volatile analytes could be helpful to resolve such a problem [9, 10]. By using the ratio formate to acetate, a statistically significant differentiation between the quartz group and both the control and soapstone exposed groups could be observed (figure 4). Acetate was selected as a representative of the positive influence on variables for two reasons. This metabolite is frequently reported as an important parameter allowing us to differentiate between healthy volunteers and patients with different pathologies (asthma [12, 15, 43], COPD [14, 33], obesity [31], or a combination of obesity and asthma [32], as well as lung cancer [44]). The second reason is that the high acetate concentration in EBC allows a good quantification with a small error. Formate was selected as a representative of the negative influencing variables in the model for three reasons.

The first one is that a statistically non-significant and low association ($r^2 = 0.16$, $p = 0.19$) with acetate is determined for healthy controls, suggesting that the source of this metabolite is different from SCFA (acetate, propionate, butyrate). The second reason is that this metabolite can play an important role in the progression of inflammatory diseases by favouring ROS production through disrupting the mitochondrial electron transport and energy production [31]. Finally, it is generated when glyoxylate (a metabolite originating from the splitting of isocitrate, an intermediate of the tricarboxylic acid cycle) reacts with ROS [38]. An increase in formate concentration could be indicative of a metabolic adaptation to fight oxidative stress. In addition, formate has been proposed as a biomarker of nitrosative stress, as it could originate from the S-nitrosothiol catabolism, as observed in severe asthma [19].

Based on these observations, we make the hypothesis that a modification of the metabolic signature is induced by exposure to inflammatory quartz and might be observed in the EBC via a change in the formate/acetate ratio. The biological effect of quartz has been demonstrated to be related to its surface reactivity and intrinsic propensity to generate ROS and oxidative stress [45]. The inflammatory property of fine and ultrafine quartz particulate has been also demonstrated for animals [46]. In order to respond to this, multiple cellular strategies to maintain the redox balance can take place: the 'classical' up regulation of non-enzymatic antioxidants (glutathion, ascorbic acid…) or enzymes (catalase, superoxide dismutase,…), or inducing a metabolic switch through controlling the reduced nicotinamide adenine dinucleotide phosphate pool ($NAD^+/NADH$ and $NADP^+/NADPH$ redox couples) [47]. For example, under oxidative stress, bacteria tend to modify the $NAD^+/NADH$ couple in favor of the $NADP^+/NADPH$ couple [38] by shunting the normal tricarboxylic cycle (TCA) at the level of isocitrate. In addition to impede the NADH production, this metabolic shift will generate glyoxylate, which produces formate when reacting with ROS [38]. Finally, under oxidative stress conditions, different signaling, activation and influx of immune cells to the inflamed zone will also take place. All these up or down regulated pathways are energy dependent and might alter the metabolic profile [48, 49].

We think that this hypothesis is plausible as already some papers report on modifications in the metabolic response of different cells or animals exposed to silica. For example, Lu *et al* [50] exposed mice to SiO_2 nanoparticles with different sizes and observed an altered metabolite profile in the liver and serum, which was interpreted as indicative of an impairment of the tricarboxylic acid cycle (TCA) and consistent with the occurrence of oxidative stress. A study about the modification of the metabolism of macrophages following exposure to silica nanoparticles has also reported a reprogramming of the

TCA cycle [51]. Similar results have been described for human embryo kidney cells exposed to magnetic nanoparticles coated with silica [52]. Another metabolomic study on human lung cells exposed to fumed silica nanoparticles reported an alteration of glucose, lactate and amino acids levels in the culture medium [53].

We are aware that our study has some limitations. The limited number of quartz exposed volunteers did not allow an external validation with an independent group for the formate/acetate ratio to differentiate exposure to inflammatory particles from less reactive one. Whereas good reasons are to advocate for using the EBC ratio formate/acetate as a marker for quartz inflammation, the usefulness of this ratio has to be verified in other studies, in particular in view of its potential relevance in clinical diagnosis. In addition, the temporal variation of these two metabolites in EBC of healthy non-exposed volunteers should be evaluated. Finally, the presence of formate and acetate in the ambient air was not measured whereas data from Izquierdo-Garcia *et al* [54] suggest a possible contribution from this side. The identification of the origin of these different anions in EBC cannot be done based on this study. For healthy volunteers, metabolic profiles for plasma, EBC and saliva have shown to be comparable, with the strongest correlation reported between EBC and saliva [55]. Compared to EBC levels, saliva contains 10–100 times larger concentrations of acetate [43]. By analogy to acetate, some other microbiome-derived anions like SCFA could also partly derive from the saliva rather than from the lungs. The observed ratio of formate/acetate in our study is about 5–12 times larger than the one calculated from a metabolomics study of saliva [56]. Other ratios calculated from this reference (lactate/acetate, propionate/acetate), differ also from the one determined in this study, suggesting that the saliva is not a strong contributing factor in the EBC anion determined in this study. The presence of a microbiota in the lung is now largely accepted [57] and its metabolism could be at the origin of SCFA in EBC. Disruption of this microbial community by exposure to reactive particles could also favour more resistant microbes, inducing that this was a modification of the secreted SCFA or other metabolites.

Conclusions

By using a validated ion chromatography method and statistical treatment based on principal component analysis, we demonstrated that exposure to a recognized inflammatory particle (quartz) induced an altered pattern of anions in EBC samples. This was reflected in the ratio formate/acetate, which was statistically larger for the quartz group compared to the group of workers exposed to the less reactive soapstone particles. This observation combined with literature data suggest that

the simultaneous measurement of different anions in EBC could be very interesting in evaluating the possible metabolic changes due to exposure to PM. In addition, the combination of IC with other techniques like NMR could be complementary and could provide deeper insight into metabolic responses to exposure to PM. Nevertheless, our results have to be confirmed in further studies.

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Conflict of interest

The authors declare no conflicts of interests.

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