Metal dyshomeostasis based biomarkers of lung cancer using human biofluids

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Lung cancer (LC) is one of the most common causes of cancer-related deaths on the world and it is well known that trace elements play important roles in the carcinogenic process activating and inhibiting enzymatic reactions and metalloproteins, in which they usually participate as cofactors. A cross-sectional study was conducted on 48 lung cancer patients and 39 controls (56 men and 31 women), aged 44-76 years between March 2011 and June 2012. Eleven elements have been included in the study: V, Cr, Mn, Fe, Co, Cu, Zn, Se, Mo, Cd, and Pb, some of them considered toxic (V, Cd, Cr and Pb), while others are essential (Co, Mo, Se, Fe and Zn), and they have been analyzed by ICP-QQQ-MS in serum, urine and for the first time in bronchoalveolar lavage fluid (BALF). In order to deepen the involvement of metal in this process, an analytical metallomic approach based on non-denaturing precipitation of proteins (NDPP) has been optimized for the fractionation of high molecular mass (HMM) and low molecular mass (LMM) metal species, in order to distinguish between metal species that affect the biological activity and toxicological potential of the elements. In this work, the NDPP followed by the analysis of metals by ICP-QQQ-MS has been applied for the first time to serum, urine and BALF samples from lung cancer patients and controls in order to get metal-size molecule profiles (MSMP), which can be used as metal-based biomarkers of altered metabolic processes as oxidative stress and homeostasis. In this sense, we have demonstrated that several metals are good biomarkers when they are related to the labile complexes, complexed with low molecular mass ligands, or in the form of metalloproteins (i.e. V and Cr in HMM and Cu in LMM), which has been described for the first time. On the other hand, metal dyshomeostasis biomarkers are proposed using metals ratios and correlations. Finally, ratios between elements resulted to be important biomarkers for lung cancer disease in serum (V/Mn, V/Pb, V/Zn, Cr/Pb), urine (Cr/Cd, Mn/Cd, V/Cd, Co/Cd, Cd/Pb) and BALF (V/Cu), which reflect the dyshomeostasis of metals in lung cancer disease. In this sense, several metals are correlated to others suggesting also the existence of an interconnected homeostasis in lung cancer.

Introduction

Elements are essential to regular human homeostasis and play significant roles in biological systems participating in many cellular processes. These essential metals, which are only required in trace amounts, are crucial for the function of numerous enzymes required for fundamental biochemical processes. For instance, iron is essential for the function of ribonucleotide reductase and zinc is a constituent of over 300 enzymes that play vital roles in gene expression. Apart from their role in enzyme function, metals are also required for numerous biological processes such as the transport of oxygen in the blood, which is mediated by hemoglobin containing iron.

Deficiency or excess of any of these elements can lead to disease (e.g. anemia) or deleterious toxic effects, inflammation and cancer. As a consequence the levels of these essential metals have to be carefully balanced and a homeostatic state is maintained within the body.

On the other hand, a number of non-essential elements can also have important implications on human health. In this way, environmental exposure to arsenic, cadmium, lead and nickel has carcinogenic consequences due to activation of oncogenic signaling pathways, oxidative stress or inhibition of DNA repairing system by Ni. Many of these non-essential metals can also alter some enzymes function, as in the case of the competitive interaction of zinc and cadmium in many enzymes as consequence of their very similar atomic structure, which has dramatic effects on many zinc-containing enzymes involved in important biological processes leading to cancer onset. Many metals also contribute to cancer progression and metastasis.

Therefore, the study of the elements levels in human tissues and particularly in serum can provide interesting information about the changes occurring during the biological processes involved in the progression of diseases such as lung cancer (LC), which is the second most frequent cancer in humans and is the common cause of cancer deaths in the world. Thus, the changes in the presence of these elements can be used as biomarkers of this disease.
of some elements and the profiling of their chemical forms can reflect the status of human nutrition and metabolism and can assist to a possible early prediction of cancer onset and development.

There are two different points of view to relate the behavior of the elements in the organism during the carcinogenic process: the disturbance of the natural chemical form of the essential element in the metabolism due to cancer onset and progression and the consideration of elements involved in the carcinogenic process as a consequence of their high exposure. On the other hand, the majority of works focus on the estimation of a deficiency state or excess and into a lesser extent about the unbalance episodes in which the excess of one element affects the function of other. However, the importance of the chemical form of elements in biology is well-known. Elements can be mainly present as labile complexes or complexed with low molecular mass ligands, or in the form of metalloproteins. This difference between low molecular mass (LMM) and high molecular mass (HMM) species is very important, since it finally affects to the biological activity or toxicological potential of the element and their mobility across different biological compartments. On the other hand, the importance of metal homeostasis and metals interactions in biology has also been demonstrated and the ratio of metals (Cu/Zn) resulted to be different in serum and whole blood and pleural effusion of lung cancer patients, but the interplay of elements are rarely reported.

Many authors have reported concentrations of essential and non-essential elements in human biological samples from patients with lung cancer, such as serum, plasma, urine, serum and hair, pleural fluid and urine in order to use the alteration of lung tissue is a more representative sample of lung status than samples. In this sense, the signal of 80Se in oxygen mode ( 96SeO+) in blood has also been demonstrated and the ratios of metals (Cu/Zn) resulted to be different in serum and whole blood and pleural effusion of lung cancer patients, but the interplay of elements are rarely reported.

Materials and methods

Instrumentation

Elemental analysis was performed by inductively coupled plasma mass spectrometry equipped with a triple quadrupole, using the Agilent 8800 Triple Quad (Agilent Technologies, Tokyo, Japan), with helium and oxygen of high-purity grade (>99.999%), and pure hydrogen gas (>95%). Instrumental conditions were optimized using a Tuning aqueous solution containing Li, Co, Y and Tl at 1 µg L−1. Nickel sampling and skimmer cones were employed, with a sampling depth of 10 mm. The forward power was set at 1550 W, and the gas flow rates were fixed at 15 L min−1 for plasma gas and 1.08 L min−1 for carrier gas. To analyze most elements a flow of 4.5 mL min−1 of helium was used. For selenium, a flow of 2 mL min−1 of H2 with 40% of O2 was used in MS/MS mode. Isotopes monitored were 51V, 53Cr, 55Mn, 57Fe, 63Cu, 64Zn, 65Cu, 66Zn, 78Se, 80Se, 95Mo, 99Mo, 103Rh, 112Cd, 114Cd and 208Pb with dwell time of 0.3 s per isotope. A MARS microwave oven (CEM Matthews, NC, USA) was used for the mineralization of samples in PFA Teflon vessels.

Standard solutions and reagents

Acetone (Trace Analysis Grade), nitric acid (purity 67-69%, Trace Metal Grade) and hydrogen peroxide (purity 30-35%, Optima Grade) were purchased from Fisher Scientific (Leicestershire, UK). Water was purified with a Milli-Q Gradient system (Millipore, Watford, UK).

Samples collection

Blood samples were collected from lung cancer patients (LC), patients with non-cancerous lung diseases (NCC) and healthy people used as control (C), at the Pneumology Area of Juan Ramón Jiménez Hospital (Huelva, Spain). The blood samples were obtained by venipuncture of the antecubital region, after 8 hours of fasting, and collected in BD Vacutainer SST II tubes with gel separator and Advance vacuum system. The samples were immediately cooled and protected from light for 30 minutes to allow clot retraction. After centrifugation (2000 g for 10 minutes) serum samples were frozen at −80°C until analysis. Bronchoalveolar fluid samples were obtained by instillation and subsequent aspiration of saline solution into one or more pulmonary segments or subsegments through a bronchoscope from patients with LC and NCC. It is not possible

"Journal Name", 2012, 00, 1-3 This journal is © The Royal Society of Chemistry 20xx

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to obtain bronchoalveolar lavage samples from healthy individuals as it is an invasive technique. The resulting liquid was aliquoted into Eppendorf tubes and stored at -80 °C until analysis. Finally, urine samples were collect in sterile urine vessels and were aliquoted into Eppendorf tubes and stored at -80 °C until analysis.

The study was performed in accordance with the principles contained in the Declaration of Helsinki and approved by the Ethical Committee from Juan Ramón Jiménez Hospital and University of Huelva. Table 1 showed clinical characteristics of patients (LC and NCC) and healthy controls (HC). In addition, all people gave informed consent for the extraction of peripheral venous blood and BALF.

Fractionation of samples and total elements determination

The fractionation of samples was based in the precipitation of proteins in non-denaturing conditions to obtain the HMM fraction (pellet) and the LMM fraction (supernatant). To this end, we used a method previously developed by the authors with some modifications 40. This fractionation procedure was only applied to serum samples since the precipitation of proteins was negligible in bronchoalveolar lavage fluid and urine samples. Briefly, 600 µL of cold acetone (-20°C) was dropwise added to 300 µL of serum and kept for 10 min in an ice bath. The mixture was vortexed and the precipitate was removed by centrifugation (12857 g, 4°C, 5 min). The supernatant, containing LMM species, was taken to dryness under nitrogen stream and reconstituted in 1 ml of ultrapure water with 0.1 µg L⁻¹ of Rh as internal standard. On the other hand, the precipitate was subjected to microwave assisted acid digestion for the determination of metal content in the HMM fraction. To this end, the precipitate was introduced into the microwave vessel together with 500 µL of a mixture containing nitric acid and hydrogen peroxide (4:1 v/v). Mineralization was carried at 400 W, ramping the temperature to 150°C in 10 min. Then, extracts were made up to 3 ml with ultrapure water, adding 0.1 µg L⁻¹ of Rh. Before analysis, samples were filtered through 0.45 µm pore size filters of PTFE.

The fractionation procedure was validated using an aqueous solution of bovine serum albumin standard containing Cu and Zn, in order to ensure the integrity of the metal protein binding during sample treatment as the same procedure used by González-Domínguez et al. 41. In addition, a reference material of serum and urine (Clinchek, Serum Control lyophilized for trace elements, level II, Recipe, and Clinchek, Urine Control lyophilized for trace elements, level II, Recipe) were used to validate the analytical method by ICP-OES-MS including the reproducibility (supplementary material 1, table 1 and 2). Finally, total metal content of serum, urine and BALF (TOTAL) was determined in diluted samples previously described 42. In this way, serum, urine and BALF samples were five-fold diluted with ultrapure water and 0.1 µg L⁻¹ of Rh was added as internal standard.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Characteristics</th>
<th>LC</th>
<th>NCC</th>
<th>HC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of samples</td>
<td>n=48</td>
<td>-</td>
<td>n=39</td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>65 ± 11</td>
<td>-</td>
<td>58 ± 14</td>
<td></td>
</tr>
<tr>
<td>Sex (M/W)</td>
<td>39/9</td>
<td>-</td>
<td>17/22</td>
<td></td>
</tr>
</tbody>
</table>

**Table 1.Clinical Characteristics of patients (LC and NCC) and healthy controls (HC)**

Statistical analysis

Statistical calculations were made in STATISTICA 8.0 software (StatSoft, Tulsa, USA). Non parametric methods were used since most of the variables showed a skewed distribution (checked by normal probability plots) and variances were not homogeneous (checked by Levene’s test). Thus, group comparison was conducted using Krustal-Wallis one-way analysis of variance, and when significance effects were observed, Mann Whitney U test was carried out for pairwise comparisons to find differences between groups. Only p values below 0.05 were regarded as statistically significant. Finally, to evaluate the specificity and sensitivity of metabolites altered by the disease, ROC (receiver operator characteristic) curves were applied to the dataset and metabolites with “area under
the curve” (AUC) higher than 0.75 were considered as relevant in the progression of LC.

Results and discussion

Mass balance of serum fractions

Mass balance evaluation for the LMM-HMM study was carried out by comparison of total metals concentration as the sum of LMM and HMM obtained from serum against the direct determination of metals in serum samples. Results ((HMM+LMM)/Total) ranged from 83 % (V) to 114 % (Mn). On the other hand, HMM obtained from serum samples accounts for the majority of the metal content and ranged from 60% (Zn) to 110% (V), while metals in LMM fraction were from below detection limit (V and Co) to 10 % (Mn) for almost all the elements. However, the LMM fraction accounts for higher percentages against the total of Zn (37%) and Cr (20%).

Classification analysis using elements concentrations in urine, BALF, serum and serum fractions

In this work, the total concentration of 11 elements in 87 serum samples, 87 urine samples and 55 BALF samples from patients with LC and controls (HC or NCC) were determined by ICP-QQQ-MS. In addition, the analysis of metal content by size-fractionation in serum (HMM and LMM fractions) provided new contributions to the characterization of the metal profile of patients with LC.

In order to establish a classification of the study groups on the basis of their elements concentrations, statistical discriminant analysis of partial least squares (PLS-DA) was performed. In addition, the weight of each variable (elements concentrations and inter-element ratios) of each biofluid (Total serum, HMM and LMM fractions, urine and BALF) in the classification (VIP value, variable importance on the projection) was used together with other criteria (see next section) for the selection of biomarkers for lung cancer.

PLS-DA is a supervised method that provides statistical models that allow visualizing groupings and trends between different groups of samples through representation of score plots.

Figure 1A represents the 3D score plot of serum samples from LC and C groups. A clear separation of the groups can be observed indicating that there are elements whose concentrations are clearly altered in lung cancer, or maybe that when a threshold concentration in the body is exceeded, lung cancer is induced.

In addition, the position of signals on the “loading” chart indicates if the element concentration is overexpressed or inhibited in lung cancer. In this way, Figure 2 shows the representation of the loadings plot obtained using the elements concentration in the HMM fraction of serum samples. Then, the signals clustered in the left side of the graph represent metals overexpressed in lung cancer, while those found in the opposite corner are reduced in these samples. Thus, the concentration of vanadium and chromium in the HMM of serum are higher in lung cancer patients, which confirm results obtained by Mann-Whitney tests. The loadings plots were also obtained for the concentration of elements in the LMM fraction of serum, serum, urine and BALF (Supplementary Material 1, figure 2, 3, 4 and 5).

Elements concentrations and proposal of lung cancer biomarkers

In order to classify the study groups, statistical discriminant analysis of partial least squares (PLS-DA) was performed. In addition, the weight of each variable (elements concentrations and inter-element ratios) of each biofluid (Total serum, HMM and LMM fractions, urine and BALF) in the classification (VIP value, variable importance on the projection) was used together with other criteria (see next section) for the selection of biomarkers for lung cancer.

Figure 1. 3D score plot obtained by PLS-DA in serum samples using the concentration of elements as variables. A) TOTAL serum, B) HMM fraction, C) LMM fraction. Red triangles: Cancer group, Black triangles: Control group.

Figure 2. Loadings plot for comparison of elements concentration in the HMM fraction of serum obtained from LC and HC.
included in the tables in order to select the most powerful lung cancer biomarkers in this paper. However, data corresponding to all the elements and ratios in serum, serum fractions, urine and BALF are collected in Supplementary Material 2.

Concentration of elements for LC and HC patients in serum samples and serum fractions (HMM and LMM) are summarized in Table 2. Total levels of vanadium in serum, vanadium and chromium in HMM fraction of serum and copper in LMM fraction of serum were statistically different between LC and C and all of them were increased in LC patients from 2 to 5-fold (V-HMM).

In this study, vanadium concentration was 3.77 and 5.03-fold higher in LC patients in serum and HMM fraction of serum, respectively. There is very scarce information about the role of vanadium in the body. Lin et al., analyzed vanadium in plasma from women with LC and found no significant differences between cancer and control groups59, that has also been concluded in other works59. On the other hand, some authors have reported the anticancer properties of vanadium and showed that complexes of these metals are the new metal-based drugs used in the treatment of several cancers, such as lung cancer46. Other authors have demonstrated the influence of vanadium compounds in the cytotoxicity of some ligands in human lung cancer cultured cells44.

Cadmium levels in urine, serum and blood of smokers has previously proposed as a marker for the development and progression of lung diseases60 and it has also found to be increased in the urine of cancer patients60,29. The National Report on Human exposure to Environmental Chemicals from the Centers for Disease Control and Prevention (CDC) reported a mean cadmium concentration of 0.172 µg/L (0.199 µg/g creatinine) for non-smokers versus 0.308 µg/L (0.336 µg/g creatinine) for smokers in the U.S. population (not stratified by age)61.

On the other hand, lung cancer patients have manganese concentrations in bronchoalveolar lavage fluid 1.5-fold higher than control group (Table 4). Manganese superoxide dismutase (Mn-SOD), as a single superoxide radical scavenger in mitochondria, may have a big role in preventing cells as an antioxidant and tumor suppressor52. In the lungs, Mn-SOD is considered to be of critical importance for antioxidant defense52. A number of studies have defined associations between the Mn-SOD Ala16Val polymorphism and different cancer types54-56. Lin et al26 found higher levels of Mn in serum from LC group according our results. In the same way, Tan et al. reported a decrease of this element in urine of LC patients29. Some authors reported decreased levels of manganese in BALF from patients with diffuse lung diseases44 or calves with mycoplasma bronchopneumonia57, but there is no antecedent about the concentration of manganese in BALF samples from LC patients.

Metal dyshomeostasis in lung cancer

Inter-elements ratios. As has been commented, lung cancer is associated with imbalances in the levels of elements that are reflected in the results from serum, urine, BALF and/or fractions (Table 2, 3 and 4). In addition, the concentrations of elements were further analyzed to obtain inter-element ratios in order to understand the interrelations of elements. Those ratios that showed significant changes between the study groups are listed in Table 2, 3 and 4 for serum, urine and BALF, respectively, which allows discovering the effect of alterations of single elements on the homeostasis of the rest in each level of structural organization. Good separations between LC and C or NCC group were also obtained using the inter-element ratios by PLS-DA in BALF (Figure 3), urine and serum samples (Supplementary Material 1, figure 6 and 7).

Results obtained shows clear interactions of V with Mn, Pb and Zn, Cr with Pb in serum, Cd with Cr, Mn, V, Co and Pb in urine and V with Cu in BALF. Several papers describes the interactions between Cu and Zn, concluding that high Cu:Zn ratios are common in serum of patients with lung cancer21-23,58-60, but we do not find this interaction to be significate using VIP, p-value, AUC and fold change criteria. The ratio between Cd and Zn has also been found to be different between smokers and nonsmokers and it is also different in smokers for several diseases and cancers60. On the other hand, the inter-element ratios that resulted to be significate different in lung cancer patients against healthy people in this paper have not been previously described and show high values of VIP, fold changes AUC and p-value.
Table 2. Total elements concentration (ng g⁻¹) in serum samples (TOTAL) and serum fractions (HMM and LMM).

<table>
<thead>
<tr>
<th></th>
<th>V (TOTAL)</th>
<th>V (HMM)</th>
<th>Cr (HMM)</th>
<th>Cu (LMM)</th>
<th>V/Mn</th>
<th>V/Pb</th>
<th>V/Zn</th>
<th>Cr/Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium concentration HC</td>
<td>0.05</td>
<td>0.04</td>
<td>0.39</td>
<td>33.33</td>
<td>0.02</td>
<td>0.03</td>
<td>0.00004</td>
<td>0.35</td>
</tr>
<tr>
<td>aSEM in HC group</td>
<td>0.02</td>
<td>0.02</td>
<td>0.17</td>
<td>2.78</td>
<td>0.01</td>
<td>0.01</td>
<td>0.00002</td>
<td>0.14</td>
</tr>
<tr>
<td>Medium concentration LC</td>
<td>0.18</td>
<td>0.19</td>
<td>0.83</td>
<td>76.29</td>
<td>0.08</td>
<td>0.14</td>
<td>0.00164</td>
<td>0.96</td>
</tr>
<tr>
<td>bSEM in LC group</td>
<td>0.04</td>
<td>0.05</td>
<td>0.12</td>
<td>2.58</td>
<td>0.01</td>
<td>0.02</td>
<td>0.00004</td>
<td>0.19</td>
</tr>
<tr>
<td>Fold Change (LC/HC)</td>
<td>3.77</td>
<td>5.03</td>
<td>2.11</td>
<td>2.29</td>
<td>3.55</td>
<td>4.27</td>
<td>4.10</td>
<td>2.79</td>
</tr>
<tr>
<td>VIP</td>
<td>1.82</td>
<td>1.72</td>
<td>1.89</td>
<td>2.08</td>
<td>1.47</td>
<td>1.47</td>
<td>1.50</td>
<td>1.64</td>
</tr>
<tr>
<td>p-value (Mann Whitney U test)</td>
<td>0.00004</td>
<td>0.000007</td>
<td>0.00003</td>
<td>0.00000</td>
<td>0.000033</td>
<td>0.00002</td>
<td>0.00005</td>
<td>0.00003</td>
</tr>
</tbody>
</table>

Figure 3. A) Loadings plot for comparison of inter-element ratios in BALF obtained from LC and NCC. B) 3D score plot obtained by PLS-DA in BALF samples using the inter-element ratios as variables. Red triangles: Cancer group, Green triangles: Control group.
Table 3. Total elements concentration (ng g⁻¹) in urine samples.

<table>
<thead>
<tr>
<th></th>
<th>Cd</th>
<th>Cr/Cd</th>
<th>Mn/Cd</th>
<th>V/Cd</th>
<th>Co/Cd</th>
<th>Cd/Pb</th>
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</thead>
<tbody>
<tr>
<td>Medium concentration HC</td>
<td>0.67</td>
<td>6.38</td>
<td>10.62</td>
<td>3.83</td>
<td>1.56</td>
<td>0.09</td>
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<tr>
<td>aSEM in HC group</td>
<td>0.07</td>
<td>1.47</td>
<td>1.66</td>
<td>0.73</td>
<td>0.24</td>
<td>0.01</td>
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<tr>
<td>Medium concentration LC</td>
<td>1.55</td>
<td>2.33</td>
<td>3.95</td>
<td>1.6</td>
<td>0.42</td>
<td>0.23</td>
</tr>
<tr>
<td>bSEM in LC group</td>
<td>0.21</td>
<td>0.47</td>
<td>0.48</td>
<td>1.43</td>
<td>0.05</td>
<td>0.03</td>
</tr>
<tr>
<td>Fold Change (LC/HC)</td>
<td>2.32</td>
<td>0.37</td>
<td>0.37</td>
<td>0.42</td>
<td>0.27</td>
<td>2.47</td>
</tr>
<tr>
<td>VIP</td>
<td>1.70</td>
<td>1.81</td>
<td>2.07</td>
<td>1.48</td>
<td>1.90</td>
<td>1.57</td>
</tr>
<tr>
<td>p-value (Mann Whitney U test)</td>
<td>0.00001</td>
<td>0.000006</td>
<td>0.000002</td>
<td>0.00002</td>
<td>0.0000</td>
<td>0.001</td>
</tr>
<tr>
<td>AUC</td>
<td>0.79</td>
<td>0.76</td>
<td>0.77</td>
<td>0.75</td>
<td>0.81</td>
<td>0.74</td>
</tr>
</tbody>
</table>

Table 4. Total elements concentration (ng g⁻¹) in bronchoalveolar lavage fluid.

<table>
<thead>
<tr>
<th></th>
<th>Mn</th>
<th>V/Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Medium concentration NCC</td>
<td>0.46</td>
<td>1.03</td>
</tr>
<tr>
<td>aSEM in NCC group</td>
<td>0.06</td>
<td>0.10</td>
</tr>
<tr>
<td>Medium concentration LC</td>
<td>0.69</td>
<td>0.51</td>
</tr>
<tr>
<td>bSEM in LC group</td>
<td>0.09</td>
<td>0.11</td>
</tr>
<tr>
<td>Fold Change (LC/NCC)</td>
<td>1.51</td>
<td>0.49</td>
</tr>
<tr>
<td>VIP</td>
<td>1.29</td>
<td>1.22</td>
</tr>
<tr>
<td>p-value (Mann Whitney U test)</td>
<td>0.003</td>
<td>0.02</td>
</tr>
<tr>
<td>AUC</td>
<td>0.75</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Table 5 shows Spearman’s correlation coefficients calculated to evaluate the interdependence of metals in the organism, suggesting the existence of an interconnected homeostasis. In this sense, levels of Cr, Mn, Fe, Pb, V, Zn, Co and Se in the different analyzed samples present a correlation coefficient between them higher than 0.5, indicating a possible common route for their biological regulation. Among these elements, Cr, Mn and V also show signficate imbalances in the analyzed biofluids (Table 2). On the other hand, the interrelations between Cr and Pb (urine) and V and Pb (BALF) were also important as can be concluded by the inter-elements ratios (serum, Table 2).

Other elements that are interconnected only by the correlation coefficients are selenium and zinc (BALF). The antioxidant properties of selenium are well-known and alterations of this element can be related with oxidative stress. In this work, selenium is positively correlated with zinc in BALF samples, which is implicated in glucometabolic disorders. Cobalt and copper are also positively correlated in BALF. The function of cobalt in the body is to be a cofactor of vitamin B12, but in the form of labile complexes is able to generate reactive oxygen species, such as copper and iron. On the other hand, other correlations have not been previously described.

Table 5. Correlations between metals in biofluids and fractions (p<0.05)

<table>
<thead>
<tr>
<th>Correlation coefficients (r) of Spearman’s test</th>
<th>0.76</th>
<th>0.78</th>
<th>0.78</th>
<th>0.92</th>
<th>0.77</th>
<th>0.76</th>
<th>0.76</th>
<th>0.76</th>
</tr>
</thead>
</table>

aStandard error of the mean (n=39), bStandard error of the mean (n=48). Fold changes (Lung cancer vs healthy people), Variable importance on the projection values (VIP), p-value (statistical pairwise comparisons by Mann-Whitney U test) and AUC values of ROC curves.

Correlations between metals in lung cancer patients

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SERUM

V-total | Cr-total (r=0.62), Cr-HMM (r=0.58)  
V-HMM  | Cr-total (r=0.62), Cr-HMM (r=0.59)  
Mn-total | Zn-HMM (r=0.5), Zn-total (r=0.44)  
Zn-total | Mn-HMM (r=0.34)  

URINE

Cr | Mn (r=0.58), Fe (r=0.58), Pb (0.63)  
Mn | Fe (r=0.7), Pb (r=0.58)  
Fe | Pb (r=0.53)  

BALF

V-total | Pb (r=0.5)  
Cr-total | Zn (r=0.55), Se (r=0.52)  
Mn-total | Cu (r=0.56)  
Co-total | Cu (r=0.55), Mo (r=0.53)  
Zn-total | Cr (r=0.55), Se (r=0.68)  
Se-total | Zn (r=0.68)  

Conclusions

The present paper describes for the first time the use of triple quadrupole ICP-MS for the analysis of metals in biofluids from lung cancer patients and healthy people. This powerful analytical methodology has been combined with a careful approach based in the use of VIP values, fold changes, p-value<0.05 and AUC values to propose the most important lung cancer biomarkers.

This work shows that metal contents in serum, urine and for the first time in BALF can be used to distinguish healthy people and lung cancer patients. In addition, several metals are good biomarkers when they labile complexes, complexed with low molecular mass ligands, or in the form of metalloproteins (i.e. V and Cr in HMM and Cu in LMM), which has been described for the first time.

On the other hand, taking into account the complexity of biological systems, some important effects may be caused by the interplay of more than two elements, but such interactions are rarely reported. Finally, ratios between elements resulted to be important biomarkers for lung cancer disease (in serum: V/Mn, V/Pb, V/Zn, Cr/Pb, urine: Cr/Cd, Mn/Cd, V/Cd, Co/Cd, Cd/Pb and BALF: V/Cu), which reflect the dyshomeostasis of metals in lung cancer disease. In this sense, several metals are correlated to others suggesting also the existence of an interconnected homeostasis in lung cancer.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

The authors thank to Spanish Ministry of Economy and Competitiveness (CTM2015-67902-C2-1-P) and Regional Ministry of Economy, Innovation, Science and Employment (Andalusian Government. P12-FQM-0442. B. Callejón-Leblic thanks the Ministerio de Educación para a predoctoral scholarship FPU13/03615. Finally, the authors are grateful to FEDER (European Community) (UNHU13-1E-1611 and UNHU15-CE-3140).

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