

# Evaluation of elemental profile in distant tissues during tumor progression

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## Research Article

**Keywords:** tumor progression, essential mineral elements, Lewis Lung Carcinoma, elemental distribution, SR-XRF

**Posted Date:** May 31st, 2022

**DOI:** <https://doi.org/10.21203/rs.3.rs-1705952/v1>

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## RESEARCH

# Evaluation of elemental profile in distant tissues during tumor progression

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## Abstract

**Background:** Knowing how cancer develops, progresses, and spreads (metastasis) is required to mobilize the development of new ways to prevent, detect, diagnose and treat cancer. Essential elements present functions in tumor progression, promoting proliferation, migration, among other protumoral cellular processes.

Understanding how those elements are related to the processes of tumor progression is of great relevance for research. The study of the elemental profile in distant tissues, which can be modulated by tumor cells to promote metastasis, has not been sufficiently investigated. Therefore, the main objective of this study is to evaluate the distribution of medium to heavy elements during tumor progression, focusing on the tumor tissue and distant tissues that could be affected.

**Results:** With this work, it was possible to observe significant changes in the concentrations' distribution of P, S, K, Ca, Mn, Fe and Cu in distant tissues caused by the presence of tumor cells. It was also possible to detect a greater similarity between tumor tissue (which has the lung as tissue of origin) and a tissue of non-origin, such as the liver, which is an unprecedented result. Moreover, changes in the distributions of concentrations were detected and studied over time for the different tissues analyzed, such as primary tumor, liver and lung, in Control and Tumor groups. Finally, the importance of elements for biological processes of normal cells, as well as tumor cells during tumor progression was also discussed.

**Conclusions:** This work presents as contributions the study of the elemental profile in tumor and distant tissues. Furthermore, this work evaluates several elements of known biological importance (P, S, K, Ca, Mn, Fe, Cu, and Zn) allowing the study of different biological processes involved in cancer.

**Keywords:** tumor progression; essential mineral elements; Lewis Lung Carcinoma; elemental distribution; SR-XRF

## 1 Background

Essential mineral elements are crucial for life, being necessary for numerous biological processes in the human body. It is well known that such elements can also have functions in tumor progression, promoting its cellular division, migration, survival, angiogenesis, maintaining cellular integrity, among other processes [1, 2, 3, 4].

Knowing how cancer develops, progresses, and spreads (metastasis), including how cancer cells interact with normal cells and tissues in other parts of the body, is required to mobilize the development of new ways to prevent, detect, diagnose and treat cancer [5]. Understanding how the role of mineral elements is related to

the processes of tumor progression is of great relevance for research.

The literature presents research regarding the relationship between chemical elements and cancer. They focus mainly on the determination of elements' concentrations in different types of tumor to characterize them [6, 7]; the comparison between distinct tissues and cells, such as normal and tumor tissues [8], tumor cells and surrounding healthy cells [9], normal, benign and malignant tissues [10], primary tumor and metastases [11]; the study of elements collected from the environment that could be related to the incidence of cancer [12, 13, 14]; the investigation of antitumoral molecules [15, 16]; as well as the evaluation of techniques of elemental analysis [17, 18, 19].

However, comparative studies of the elemental profile between tumors and distant tissues are still scarce. Among them, the work of Frank *et al.* from 1986

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[20], establishes the elements' profile during tumor progression of Lewis Lung Carcinoma (LLC) cells. They observed the elements and their fluctuations during tumor growth in the tumor tissue as well as lung, liver, and skeletal muscles, through the X-Ray fluorescence technique induced by radioisotopes. The paper highlighted the presence of large fluctuations in the elements' levels throughout tumor progression, in addition to the elements profile of the primary tumor greatly resembling its original tissue (the normal/healthy lung) and being very different from other normal tissues studied.

In this context, there is currently a demand for progressing and updating the study on elemental profile in tumors and distant tissues that can be modulated by tumor cells to promote metastasis. This work addresses this demand by evaluating the elemental distribution of concentrations during tumor progression, focusing not only on the tumor tissue but also on distant tissues. The role of several elements of known biological importance (P, S, K, Ca, Mn, Fe, Cu, and Zn) are evaluated, allowing the study of different processes of cancer [2, 3, 4].

For this work, we analyzed data indicating the presence, concentration, and location of different elements in different tissues, in control and experimental (tumor-bearing) groups, obtained in 5 weeks of tumor progression. These data reveal the multielemental composition of the primary tumor and distant tissues, such as the lung and liver. In order to extract relevant information inherent to the voluminous data available, data preprocessing and statistical analyzes were performed.

The simulation of tumor progression *in vivo* was obtained via the inoculation of C57BL/6 mice (isogenic) with LLC cells (murine Lewis Lung Carcinoma). This tumor cell line has the lung as its tissue of origin and is widely used as a model of metastasis, being tumorigenic. They are known present tropism to the lungs, especially and, in some cases, the liver [21, 22, 23, 24, 25, 26]. The analysis of the biological samples was performed through synchrotron radiation X-Ray Fluorescence technique which is a non-destructive technique for biological samples and is effective for determining the presence, quantity, and location of elements in a sample. This technique is widely used in elemental analysis, especially for elements that present atomic numbers greater than 11, such as the ones analyzed in this work [27, 28].

With this work, it was possible to observe significant changes in the elemental concentrations' distribution in distant tissues caused by tumor cells. It was also possible to detect the similarity between tumor tissue and a tissue of non-origin, which is an unprecedented

result. Moreover, time-dependent elemental changes were analyzed and compared in this work for the different tissues and groups studied. Finally, the importance of elements for biological processes of normal cells, as well as tumor cells during their tumor progression was also discussed.

## 2 Methodology

This research was conducted in two phases: biological experiment ([29]) and data analysis. For the biological experiment, tissue samples of liver, lung, and primary tumor were collected from C57BL/6 mice. These animals were separated into two groups, a tumor-bearing group, from now on called Tumor group, inoculated subcutaneously with LLC cells; and a Control group, that was inoculated with vehicle. All animals were kept under standard conditions. The primary tumor (originated from the inoculation, establishment and growth of LLC cells) and the liver had their samples collected at weeks 0, 1, 3, and 5 allowing for the study of tumor progression in a time-dependent manner. The lung, the main tissue to which LLC cells migrate (already discussed in the previous section), had its samples collected only at week 5. The samples were analyzed at D09B X-ray Fluorescence beamline at room temperature and ambient pressure according to Sartore *et al.* [30]. The X-ray fluorescence technique was performed at the UVX Light Source at the Brazilian Synchrotron Light Source Laboratory (LNLS). All procedures involving animal experimentation were approved by the Federal University of Rio de Janeiro Animal Experimentation Committee (protocol number: 015/18) and were performed in accordance with the Brazilian guidelines for scientific use of animals. For more details of the biological experiment refer to the work of Stelling *et al.* [29].

### 2.1 Data Analysis

After the execution of the biological experiment, the data obtained were prepared for analysis, going through the preprocessing step. This phase involves cleaning, transformation, integration, and data selection [31].

#### 2.1.1 Data Preprocessing

The data obtained from the biological experiment contains, for each tissue, group and week analyzed, values in mass fraction for each element detected at each point/location of each sample. Data cleaning was carried out, where data referring to elements affected by artifacts, such as argon, an element within detection range and present in the atmosphere, were filtered and removed from the data for analysis. The mass fraction values were transformed to ppm concentration.

Next, data files were integrated generating a single dataset containing 13 attributes regarding Group, Tissue, Week, Sample (animal), and Point, in addition to each of the eight elements (P, S, K, Ca, Mn, Fe, Cu, and Zn). The integrated dataset features over 188,000 points detected for each element.

Finally in the preprocessing step, the data could be selected according to the research objectives. Therefore, the attributes "Sample" (animal), and "Point" were removed. The attribute "Point" was discarded since it was not possible to compare the locations of the elements in different samples. Such attribute (points) can be used in the construction of spatial distribution maps (imaging technique) however, this is not the focus of the current work. The sample attribute could be removed, considering that the animals in question are isogenic and were kept under the same conditions during the biological experiment performed.

### 2.1.2 Statistical Analysis

During the statistical analysis, groupings were created to allow comparisons within the data to be made from a broader to a more detailed level. Thus, groupings were obtained at the group level, at tissue level, and week level, as seen in Figure 1. The first one contains data from all tissues and weeks for each group, that is, control and tumor. The second one presents data from all weeks for each tissue within each group. Finally, the week level refers to data for each week in each tissue and group.

This work uses the week level grouping (more detailed grouping) for analysis to allow the observation of any changes in the distribution of elements that occur over time. Also, in order to make comparisons between all groups and tissues, week 5 (seen in the week level) was used since this week possesses data for all tissues and groups (figure 1). Thus at the week level, one can compare tissues at week 5 of each specific group, such as the liver and lung tissues of the control group, or even compare tissues from different groups, such as the liver of the control group versus liver of the tumor group. Hence, with this grouping is possible to compare weeks of different tissues and groups or even follow the weeks within the same tissue, observing changes over time. To make time-dependent comparisons, weeks that have data in both groups were used, hence weeks 1, 3, and 5.

Our research data present a non-normal distribution since the concentrations distribution of each element along the tissue is asymmetric. That is, very different concentration values can be detected because, in the same tissue sample, there can be high and very low elemental concentrations in different locations. Thus, the selection of statistical measures and relevance tests resistant to such asymmetry is necessary [32].

In order to obtain statistical summaries of the concentration data, the median and interquartile range were used. The Wilcoxon test (with 95% confidence) was chosen to analyze the statistical relevance of the differences between the concentration distributions. This test stands out from others as it is resistant to non-normal distribution and can still be used for log data [33].

Both preprocessing step and data analysis were carried out via the R language and computational environment. R is one of the main languages for statistical data analysis, including its preprocessing [34]. The data obtained from the experiment and the code used in the preprocessing and analysis of these are available at GitHub<sup>[1]</sup>.

## 3 Results

Figure 2 shows elements' concentrations distribution in the tissues (primary tumor, liver and lung) for the Control and Tumor groups at 5th week of tumor progression. This figure shows density plots corresponding to the distribution. In these plots, the greater the frequency of concentration values in that range, the greater the peak observed.

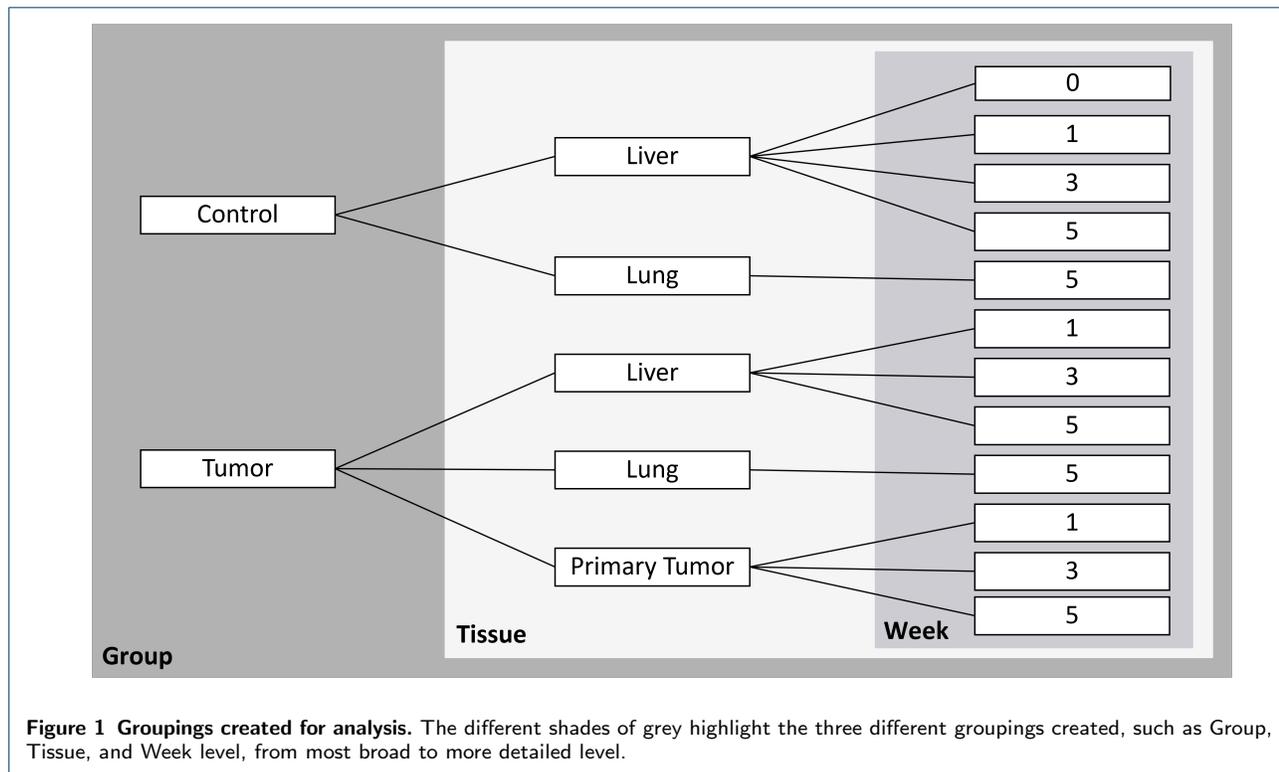
With this grouping (at the week level), it's possible to observe changes that occurred in the tissues between the two groups and it becomes possible to detect alterations in distant tissues caused by the presence of the primary tumor.

It is possible to highlight that 7 of the 8 elements obtained a statistically significant difference in their distributions between the groups. Zinc (Zn) was the only one that presented not statistically significant differences in its distributions between Control and Tumor.

When analyzing figure 2 it is noticeable that, for most elements, the distributions present considerable differences between liver and lung tissues. In addition, a greater similarity between the primary tumor and the liver can be observed when compared to the primary tumor and lung. Furthermore, when comparing the liver and lung distributions between their Control and Tumor groups at week 5, it can be observed that while the lung does not seem to be much altered, the liver appears to show more differences between Control and Tumor. Iron (Fe) also stands out since it presents greater differences in its distributions between tissues and groups. Copper (Cu), although still presents relevant differences between its tissues and groups, shows some stability in its distributions, when compared to the other elements.

In addition to observing the primary tumor against the other analyzed tissues, it was also possible to focus

<sup>[1]</sup><https://github.com/SamellaSalles/elemental-distribution>



only on the tumor tissue. Figure 3 shows boxplots that represent the distribution of each element throughout the weeks, in which the medians of concentration for each week are emphasized. Hereby, it was possible to observe elements with distinct distributions, presenting alterations in their distribution for each week. It can be highlighted the increase in the level of iron (Fe) over time, the increase in the level of potassium (K) from week 1 to 3, in addition to the elements sulfur (S) and phosphorus (P) showing similarities between their levels of concentration and behavior throughout the weeks.

Figure 4 shows the concentration distributions of the elements in the liver, throughout the weeks (1, 3, and 5), for the Control and Tumor Groups. These data were also compared with those obtained for the tumor tissue. Thus, with this figure, we can observe alterations, caused by the presence of the primary tumor, in the liver (distant tissue) over time, in addition to observing possible similarities between these two tissues.

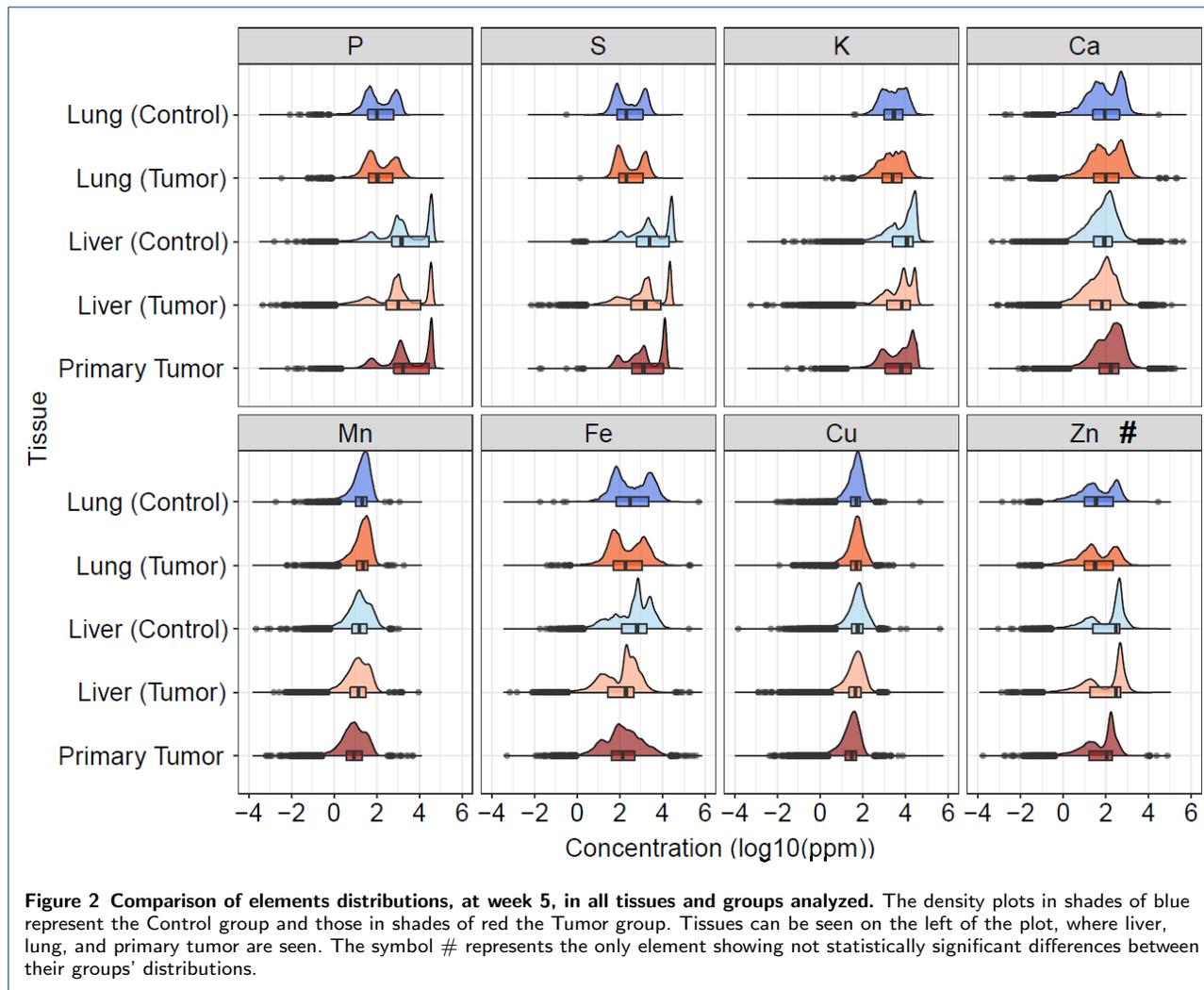
In this figure, the elements phosphorus (P) and sulfur (S) seem to have both their concentration values and distributions similar. Over the weeks, the distributions of these two elements also show similarities. With the statistical analysis performed, it was also possible to notice that zinc (Zn) presents its distributions with no statistically relevant differences in the liver, between Control and Tumor Groups, in weeks 3 and 5.

In addition to investigating how the elements had their distribution modified over time between the two groups, we also looked for similarities between the liver (Tumor Group) and the primary tumor. Interestingly, in figure 4, it was observed that potassium (K), while presenting distributions with statistically significant differences between the Control and Tumor groups, as seen in figure 2, had its distributions showing smaller differences between liver (Tumor Group) and the primary tumor over the weeks, where at week 5 there were not statistically relevant differences in the distributions between these two tissues.

## 4 Discussion

The first question we sought to answer is regarding the existence of alterations in elemental distributions in distant tissues. These distant tissues (liver and lung) are described in the literature as primary tumor targets for the formation of pre-metastatic niches that could facilitate invasion and colonization by tumor cells during metastasis.

When evaluating the differences in the distribution of elements between the groups (Control and Tumor) for the liver and lung (figure 2), it was possible to notice that, with the exception of zinc, all elements obtained statistically significant differences in their distributions.



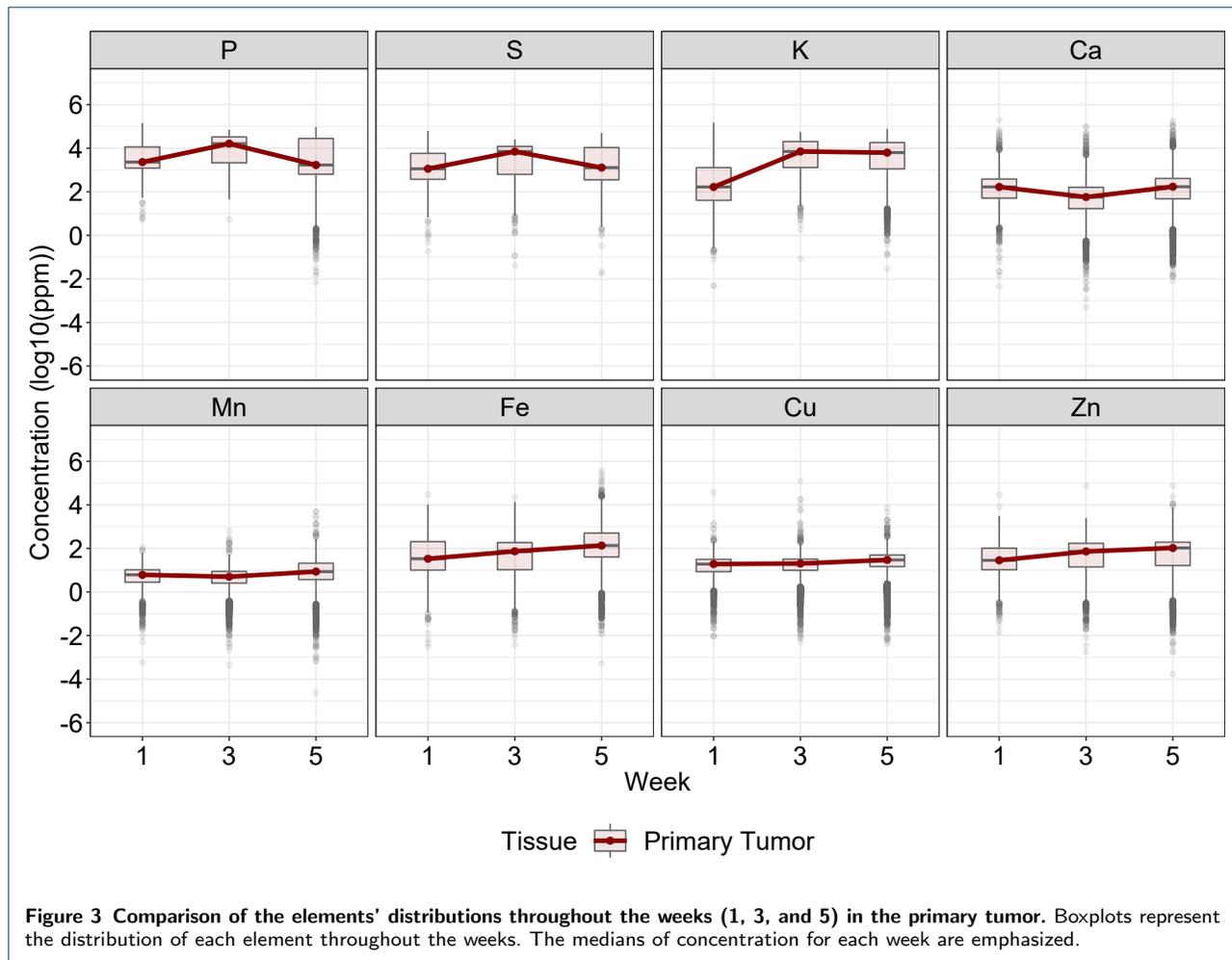
Since the most relevant difference among the analyzed groups is the subcutaneous inoculation of LLC cells that developed into a tumor (Tumor group), with this work, it is possible to observe statistically significant changes in the distribution of elements in distant tissues caused by the presence of the tumor.

According to the literature, among the liver and lung, LLC cells have a preference for the lung, when inoculated *ex-situ*, during the metastasis process, which can be attributed to its lung epithelial origin [24, 25, 26]. Interestingly, in this work, it was possible to observe statistically significant differences in the distribution of elements for these two tissues, not being highlighted a preference for tumoral modulation for a tissue in particular.

When we analyzed elements' concentrations distribution in all tissues (figure 2) at week 5, it was possible to observe a greater similarity between the liver and the primary tumor, compared to the tumoral tis-

sue and lung. This fact is unexpected due to the LLC lineage pulmonary origin, besides the similarity of this lineage type with the tissue of origin, but differences with other tissues has already been established in the work by Frank *et al.* [20]. While researching for bibliographies that address such similarity between tumoral tissue and liver, it was not possible to find such a result in other works. Thus, such a result showed to be unprecedented.

As seen in figure 2, in addition to zinc (Zn), copper (Cu) also showed fewer variations in their distributions among different groups and tissues, although copper (Cu) still showed a statistically significant difference between Control and Tumor. This makes sense, since these two elements (zinc and copper) are of great importance to cells and are closely related. These two elements have known essential functions such as: maintaining the integrity of the cell membrane; cell proliferation and growth; having antioxidant properties, by



participating in the structure of the antioxidant enzyme superoxide dismutase (SOD), guaranteeing defense against reactive oxygen species (ROS) and consequently also maintaining the integrity of the DNA; being involved in gene expression, apoptosis, among other processes [35, 36, 37, 38, 39, 40, 41, 42, 43, 44]. Due to such functions and their importance, the level of these elements and their transport are tightly regulated [2].

In contrast with the two elements above, iron was an element that showed noticeable variations in its distributions over the weeks for groups and tissues. Despite each tissue having its specific need for this element, in the figures 2 and 4 a greater variation in iron distributions between the Control and Tumor groups of the liver can be seen, in addition to an apparent increase of this element, over the weeks in the tumor tissue, also observed in the figure 3.

Iron is essential in a vast number of biological phenomena [2]. This element controls cell proliferation and growth by regulating DNA synthesis, besides having a

role in the tumor microenvironment, angiogenesis and metastasis [45, 1, 46, 47]. Therefore, this element is important for tumor progression, the formation of pre-metastatic niches and the colonization of tissues.

Since tumor cells present excessive proliferation rates, it is believed that tumor cells require higher concentrations of this element. Consequently, higher concentrations of iron are often observed in tumor tissues, in which there are increases and decreases in the expression of proteins involved in iron uptake and its efflux [48, 46, 49, 45]. This increase can be seen in figures 3 and 4, where the median of iron concentrations (seen in the boxplot) increase over the weeks.

When we focus on the primary tumor (figure 3), it is important to highlight that every tissue has its specific needs and functions, which may require different concentrations and distributions of elements, even throughout the weeks, specially during tumor progression [50].

As seen in figure 3 greater differences can be observed between the distributions over the weeks. This result is

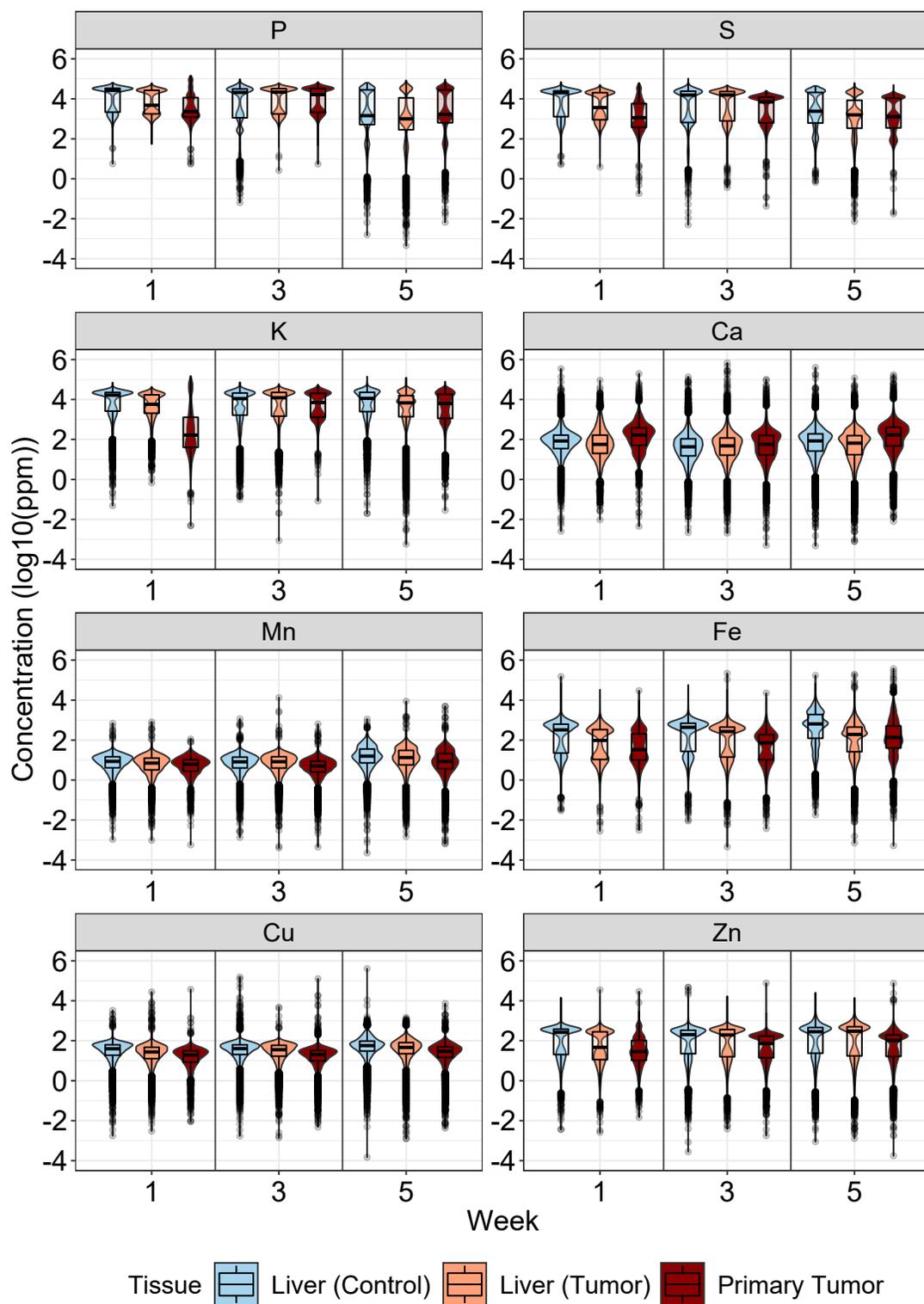


Figure 4 Comparison of the elements' distributions throughout the weeks (1, 3, and 5) in the liver (Control and Tumor groups) and primary tumor. Boxplots and violin plots in which light colors represent the liver, with light blue showing the Control group and light red the Tumor group. The primary tumor is represented in dark red.

consistent with the literature and with what has been described, since the tumor tissue is developing, adjusting to its environment and reciprocally modulating it [51]. Thereby, the changes in the distribution of the elements in this tissue are part of the stages of tumor progression, such as: the increase in iron (Fe) levels throughout time within this tissue [45]; the increase in zinc (Zn) levels, which is already known to be captured from the circulation by tumor cells to maintain their growth and the integrity of their membrane [41, 43]; in addition to the increase in potassium level in the initial weeks of tumor progression, as will be explored next [52].

In figure 4, when we focus on the liver and compare it with the primary tumor, looking for signs of alterations in this distant tissue over time, an element stands out. The statistical analysis performed showed that potassium (K) had its distributions with statistically relevant differences between the Control and Tumor groups in all weeks analyzed. However this element had its distributions between liver (Tumor Group) and primary tumor showing smaller differences over the weeks (figure 4), obtaining no statistically relevant differences at week 5. This fact is interesting since the tumoral tissue could be modulating the levels of potassium in the distant tissue (liver) during tumor progression to levels different of Control, but similar to the primary tumor in itself. The importance of this element for cell functions and tumoral progression, especially for metastasis could explain its modulation in the tissue.

When discussing potassium role, it is essential to acknowledge potassium ion channels (the most abundant type of ion channel)[53, 54]. These channels are crucial for the proper functioning of cells, including tumor cells, besides being important for the metastatic process being involved in: cell mobility; loss of cell-cell contact; interaction of cells with their environment, a process that is also relevant for tumor progression since cancer cells can modulate their microenvironment, as well as distant tissues, as seen in pre-metastatic niches; regulation of cell volume; apoptosis; angiogenesis [53, 54, 55, 56, 51]. Furthermore, it has already been established that integrins (adhesion proteins relevant to cell migration) and potassium channels are profoundly correlated [57].

Moreover, in several types of tumor cells, the expression of these potassium channels, as well as genes involved in their production, was observed to be increased, especially in the early stages, due to their importance in cell proliferation [52, 53, 58]. This may explain the rapid increase in potassium levels at the primary tumor at weeks 1 to 3 seen in figures 3 and 4.

Finally, two elements presented a similar behavior (as seen in figures 3 and 4), with concentrations distributions showing similarities even over the weeks. Those were phosphorus (P) and sulfur (S). The function of these elements can explain such similarities, since both P and S are extremely necessary for cell division and, for tissues that undergo rapid cell division, such as tumor tissue, this process is continually required [59, 60]. Phosphorus is an essential element for nucleic acid, phospholipids and high-energy molecules, such as ATP, synthesis. Sulfur is necessary for the synthesis of amino acids such as methionine, cysteine, among others [61, 62].

## 5 Conclusion

Although the importance of essential mineral elements in biological processes and tumor progression is well established in the literature, there is a scarcity of studies focusing on the investigation of distant tissues that could be conditioned by tumor cells to promote metastasis. In this context, the main objective of this study was to evaluate the elemental distribution of concentrations of several elements of known biological importance during tumor progression, focusing not only on the tumor tissue but also distant tissues.

This paper allowed the detection of significant changes in the elemental distribution of concentrations in distant tissues caused by the presence of a primary tumor, which could indicate a new aspect of pre-metastatic niche formation. It was also possible to observe the similarity between primary tumor and a tissue of non-origin, which is an unexpected and unprecedented result. Furthermore, it was possible to analyze and compare the elements' concentrations distribution in a time-dependent manner for different tissues, such as primary tumor, liver, and lung, in Control and Tumor groups. The importance of elements for the biological processes of normal cells, as well as tumor cells during tumor progression, was also highlighted.

Perspectives of the present work include further investigations on the role of essential mineral elements in the formation of pre-metastatic niches. It is also our goal to explore the elemental signature presented by the primary tumor, as well as the greater similarity presented by the primary tumor with a tissue of non-origin.

The role of essential mineral elements as modulators of the tumor microenvironment and cell fate is a relevant aspect of tumor progression and this work is a contribution to this field. The description of elemental distribution patterns from an *in vivo* model of tumor progression described in this work highlights the complexity of tumoral metallomics and ionic equilibrium.

### Acknowledgements

This research's biological experiment used resources of the Brazilian Synchrotron Light Laboratory (LNLS), an open national facility operated by the Brazilian Centre for Research in Energy and Materials (CNPEM) for the Brazilian Ministry for Science, Technology, Innovations and Communications (MCTIC).

### Funding

The biological experiment and analysis and interpretation of data were supported by CNPq, CAPES, FAPERJ, and IFRJ.

### Abbreviations

LLC: Lewis Lung Carcinoma; LNLS: Brazilian Synchrotron Light Source Laboratory; SOD: Superoxide Dismutase; ROS: Reactive Oxygen Species.

### Availability of data and materials

The datasets and code used for data preparation and analysis are publicly available in the GitHub repository (<https://github.com/SamellaSalles/elemental-distribution>).

### Ethics approval and consent to participate

All procedures involving animal experimentation were approved by the Federal University of Rio de Janeiro Animal Experimentation Committee (protocol number: 015/18) and were performed in accordance with the Brazilian guidelines for scientific use of animals.

### Competing interests

The authors declare that they have no competing interests.

### Consent for publication

Not applicable.

### Authors' contributions

M.P.S., S.C.C., and M.S.G.P. conducted the biological experiment. S.S. performed the data preparation and analysis, with R.S. as a contributor. S.S. wrote the manuscript. M.P.S., S.C.C., and R.S. did the writing review. All authors read and approved the final manuscript.

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