Author's response to reviews

Title: Aluminum concentrations in central and peripheral areas of malignant breast lesions do not differ from those in normal breast tissues

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Author's response to reviews: see over
Dear Ms. Chap,

Thank you very much for the diligent review of our manuscript, which we are pleased to resubmit in its revised form. We addressed each of the referees’ concerns carefully, and responded to their queries and demands in a point-by-point response letter. In the next pages, you will find our responses; the modifications in the text have been highlighted. Also importantly, the English language has now been professionally edited by Edanz. We hope our manuscript can now meet the standard requirements of BMC Cancer and can be deemed acceptable for publication.

We are looking forward to receiving your response.

Yours truthfully,

Luis Sarian
“Major Compulsory Revisions”

Comment 1: “The Introduction, specifically in relation to human exposure to aluminium, is weak and lacking in appropriate and seminal references. In relation to this I personally avoid citing any publications by Priest as he is funded by and is a consultant to the global aluminium industry. This was pointed out by myself to the Journal of Environmental Monitoring (7, 640, 2005) in a Letter to the Editor about the paper the authors have cited [1].”

Response 1: Thank you very much for the diligent review of our manuscript. And thank you for pointing out the problem with Priest; we were unaware of that conflict of interest. Priest’s references have now been removed. We have now amended the introduction at several points, and cited important references in the field.

Comment 2: “Our research [4] has not been accurately cited. We showed that the Al content of the outer breast region (axilla + lateral) was higher than the inner breast region (mid+medial). Additionally, we did not make any reference to whether or not the tissue donors used Al-based antiperspirants. We did not have this information.”

Response 2: Thank you for the important clarifications. We apologize for the inaccuracies. First, we removed the text which stated that you had information on antiperspirant use. Secondly, we rephrased the sentence pertaining to reference 3 and yours, for better accuracy, as follows: “Recent evidence showed increased genomic instability in the outer quadrants of the breast [3], and one study suggests that higher levels of aluminum may be present in the outer regions of the breast (axilla and lateral) compared to the inner breast regions (medial and middle) [4].”

Comment 3: “It is really not the case that previous studies which measured the Al content of breast tissue used inferior analytical methods. For example, we used GFAAS
in our study [4]. This emphasis on the improvement in analytical technique should really be removed.”

Response 3: We agree. We toned down the text in this respect, specifically paragraph #4 of the introduction. Also, we now cite your study as using GFAAS.

Comment 4: “The Materials and Methods does not include sufficient information about the measurement of Al in the breast tissues. The authors emphasize their approach to measurement as a key part of their research and if they wish to do so then they must include much more information relating to the tissue Al measurements. By way of example, the authors might wish to consult our recent paper on measuring Al in brain tissue (Metallomics 4, 56-65 2012).”

Response 4: Indeed, our description of Al measurements lacked detail. We used your reference as a model of how to describe our technical procedures. For instance, we inserted additional text on the calibration of the equipment and the software used.

Comment 5: “The authors might note that we also did not find any significant differences between the mean Al content of the 4 breast regions in our study. However, when the data were analysed to take account of the within-individual trends it is then that significant differences between the outer and inner regions of the breast are found. Perhaps similar statistical analyses as described in our paper [4] could be carried out on this data set?”

Response 5: We agree with your suggestion. Indeed, in Table 2, the p-values were calculated using paired tests. We have now reinforced that information in the legend. From Table 3 onwards, we have now performed additional paired tests in order to evaluate whether within-individual differences occurred. For that purpose, we have now added the proportion trend test (Chi-squares for trend) in Tables 3, 4 and 5.

Comment 6: “The authors should not continue to make the somewhat spurious claim that their analytical method is superior to previous methods measuring Al in breast tissue.”
Response 6: We agree. Those statements were removed from the discussion as well as in the introduction. Several modifications were made, and we hope all unsubstantiated statements have now been removed.

Comment 7: “To my knowledge, there has not been any previous attempt to compare the Al content of ‘diseased’ and ‘healthy’ tissues taken from breast mastectomies? Our research [4] simply looked at the location of the breast tissue sample and not its ‘disease’ state. If the authors want to make this distinction then they need to make it very clear as to the exact locations of each breast tissue sample, as mentioned previously, a diagram might be helpful. I would then suggest that they then apply more sophisticated statistical methods (see our paper for an example) than simply comparisons of mean Al content to try to ascertain whether for each individual there are any trends in the distribution of Al between the sampling regions. For example, the Al content of the tumour regions could be higher in all 150 individuals but because of the heterogeneous nature of Al accumulation in tissues these findings would be obscured by simply looking at mean Al contents.”

Response 7: We are in full agreement with you in that tests used to detect within-individual trends should be used. For that reasons, we added the p-values for trends in Tables 3 onwards, as explained before. Also, thank you for the suggestion of a diagram: Figure one now shows as schematic representation of the locations from which the tumor samples were obtained.

Comment 8: “It is clearly wrong and disrespectful to previous research to suggest that there have been no previous ‘precise’ measurements of Al in breast tissue! Please remove this statement or qualify it in some way.”

Response 8: We removed these statements.

Comment 9: “This manuscript can claim to have at its disposal the largest number of tissue donors. It is now very important that the data obtained from these donors are validated and analysed using the most appropriate statistical methods.”

Response 9: We agree.
Comment 10: “It is not appropriate to compare these data with our data [4]. In the first instance the locations from which the tissues were taken do not seem to match up with those in our study. Secondly we separated the breast fat (oil) from the tissue and measured the two fractions separately. The relationship that we found was with Al associated with the defatted tissue and not with the oil. Incidentally, we have just completed a new study where we did not make this distinction and when we looked at whole tissues we did not see any differences in the regional distribution of Al, as is suggested in this study.”

Response 10: You are mentioning very important issues which we interpreted mistakenly. We have now clearly stated that your study differs from ours in that we focused our analysis on the differences between central versus peripheral regions of breast tumors, and those versus the normal surrounding tissues. Our study was designed to evaluate Al concentrations across the breast. This can now be seen at the end of the second paragraph of the discussion.

Comment 11: “The authors need to explain their comment concerning ‘calcium-aluminium compounds’!? What are these? The authors do seem to be a little confused with respect to the biological chemistry of aluminium.”

Response 11: Yes, we used incorrect concepts. We were actually referring to the calcium binding protein osteopontin which coincidentally forms stable complexes with aluminum. This has now been clarified in the text. We also cited a similar problem that occurs with iron.

Comment 12: “Overall, there is much in this manuscript which should eventually be published. However, much more detail, including the use of appropriate controls such as method blanks et c., is required concerning the measurement of Al in the tissues. We also need to be clear as to the exact location from which each tissue sample was taken. The authors should then statistically analyse the Al content of each location for each donor, perhaps in a manner similar to ourselves [4] so that important data can be obtained on whether or not there are any differences in the Al content of the different regions. Mean values for each region are simply not sufficient.”
Response 12: Thank you for the encouraging comments. You detected several important issues, which we tried to address diligently. We hope this new version can now be deemed acceptable.

“Minor Essential Revisions”

“Discretionary Revisions”

Comment 13: “A diagram might be helpful in explaining the location of different tissue samples relative to the position of the tumour. I was not able to understand fully where each sample was taken from.”

Response 13: Good suggestion. We have now included the diagram describing how we sampled the tumors (figure 1).

Comment 14: “The mean measured Al contents given as mg/kg dry wt. are very similar to those in our study [4] which varied from 1.7 to 2.1 #g/g dry wt. It is really quite easy to convert between nmol and ng, simply divide by the atomic wt. of Al.”

Response 14: Thank you for the clarifications.
“Major compulsory revisions”:

Comment 1: “The paper seems to be very critical of all previous work and the statement on lines 3-4 of the abstract is unjustified ("the lack of specificity of the techniques used up until now may have led to false conclusions") and unless justified should be removed. Some previous studies used also the technique of graphite furnace atomic absorption spectrometry as the current study and it is not made clear why all previous use of this technique was inadequate.”

Response 1: Thank you very much for the accurate review of our manuscript. We agree in that we were over-critical of previous works. More than that, we acknowledge that many criticisms were unsubstantiated. We made a thorough revision of the text, which we believe is now far less critical of previous works. Also, we have now pointed out that GFAAS has been used in previous studies using breast samples.

Comment 2: “The literature review is rather selective in citation. For example, the only epidemiological study measuring amount of antiperspirant used in relation to breast cancer is not cited (McGrath 2003. An earlier age of breast cancer diagnosis related to more frequent use of antiperspirants/deodorants and underarm shaving. Eur J Cancer prevention 12: 479-485.).”

Response 2: Excellent suggestion. This is the second reference in the new list of references.

Comment 3: “Since the data are not published in full, it is not possible to see whether the data form a normal distribution to justify the current statistical analysis or whether non-parametric analysis would have been more appropriate. It would be very interesting to simply publish the full table of raw results or to construct a figure with the range of measurements. However, at the very least, a statement that the data conform to
a normal distribution needs to be made if averages are to be used rather than median values. This in itself would be different from many other measurements made in breast tissue, not only for aluminium but organic xenoestrogens.”

Response 3: You are correct. Our description of the statistical methods was incomplete. Our raw data do not conform to normality, but the log-transformed values do. Thus, v-values were calculated using the log-transformed (to base e) data. As stated by Altman, it is possible to “…perform the calculations on the log data and transform the answers back to the original scale”. This is what we did, because displaying log-transformed aluminium concentrations would seem meaningless to the reader.

Comment 4: “It is not clear why Tables 3-5 have categorized aluminium levels into three groups or how the rationale for the three groupings was decided. Since values have been measured for each sample, these results should be re-analysed using the full range of numbers which are not artificially grouped.”

Response 4: The three groups were determined using percentiles (0-33%, 33-66%, 66%-100%). This has now been explained in the statistics section. We used this approach because, when sub-setting the sample, the homogeneity of variances was lost. Then, the comparisons of the means became invalid.

Comment 5: “The end of the second paragraph of the discussion states that “In our study, by contrast, the aluminium concentration did not differ across breast quadrants”. However, the location of the tissue samples is not clearly described in the manuscript and as far as I can read, the quadrant location is not given or any analysis described in relation to breast quadrants. I cannot even find whether the peripheral or normal tissue was sampled from the axillary or sternal side of the tumour or which quadrants the tumours were located in? It is also not clear whether the current samples were total tissue or whether fat had been removed – this is relevant if results are to be compared to reference 4.”

Response 5: In fact, the quadrants from which the samples were taken are described in Tables 3-5. However, this was not the main focus of our analyses. We were concerned about the Al differences across the regions of the tumors (central vs peripheral vs normal surrounding tissues). For clarification, a diagram showing how tissues were
sampled has now been included (Figure 1). Our samples were not de-fatted, we used vacuum desiccator instead. It is worth noting, however, that we sampled tumors and normal glandular tissue, which have low contents of fat.

“Major revisions needed but more specific”:

Comment 6: “The levels of aluminium cited in the abstract should be given with an estimate of error as in Table 2.”

Response 6: Good suggestion: error estimates now displayed also in the abstract.

Comment 7: “The statement in line 2 of the results and in the legend to Table 2 “normal portions of the tumor” does not make scientific sense.”

Response 7: Of course! Thank you for pointing this out. We meant normal breast tissues. We apologize.

“Comment for consideration by the authors”:

Comment 8: “Since the manuscript cites and critically appraises previous studies, it would be helpful in the discussion to tabulate how levels of aluminium measured here relate to levels in other studies. Agreed that values are cited in different units in different papers, but it is not hard to convert from gm to moles or to find at least an estimate of equivalents from wet tissue weight to dry tissue weight.”

Response 8: We agree. We have now inserted new text in the 3rd paragraph of the discussion, comparing the tissue aluminum contents found in different studies, after converting to the units used in our study.
Comment 1: “How the AA arbitrarily determined three groups for Al content?”
Response 1: We determined the groups based on a three-tiered percentile distribution of the raw data. This has now been explained in the statistics section.

Comment 2: “On which chemical and biological basis they select the specified cut-offs?”
Response 2: There is no biological basis. As said above, this was a decision based on the data distribution (percentiles).

Comment 3: “The possible categories should be obtained after the recognition of biopathological characteristics of breast tissues (both normal and tumors).”
Response 3: We agree, but our analysis showed that the distribution of the Al concentration did not differ across the regions examined. Thus, we used the same cutoff points for the central and peripheral regions of the tumor and for the normal breast tissues as well.

Comment 4: “No detail is described for the technique and no reference has been reported to highlight the high-specificity technique (lack of methodological issues, very high standard deviations, well-known or approved method, specificity, reliability, %CV, etc).”
Response 4: We have now included several methodological details on the GFAAS technique, and new key references were cited. Most importantly, we converted aluminum measurements made by other investigators to the units used by us, and compared those results.
Comment 5: “Before to declare that "Although the relationship between aluminum exposure and breast cancer has been hypothesized for a long time, precise assessments of the aluminum contents in the breast were inexistent up until now." the AA should read more carefully the literature, focusing attention not only on breast tissues but also on breast fluids. The literature data clearly demonstrated that Al may be accumulated in normal and cancer breast fluids. The accumulation is not related to plasma concentrations or transport."

Response 5: Indeed, after re-reading our own text, we are prone at agreeing with you in that those statements were inaccurate. For this reason, our text has now been cleaned up from that type of statement.

Comment 6: “Before to declare that previous methods of Al evaluation were "to low sensitivity-low specificity detection techniques", the AA should compare the methods or at least provide reliability of their technique in breast tissues. The AA obtained very high standard deviations!!"

Response 6: This has now been removed from the discussion.

Comment 7: “It has been previously reported also in breast fluids that Al has been found as linked to menopause and that age may have such a role in the accumulation of AL through antiperspirant use.”

Response 7: There is now new text in the 4th paragraph of the discussion which mentions those Al measurements in breast fluids. Many thanks for the suggestion.

Comment 8: “More cautious evaluation of data related to bibliographic source of Al toxicity.”

Response 8: Thank you very much for this suggestion too. We have now been informed that some authors cited in the original version of the text had very serious conflicts of interest (linked to the aluminum industry). We have avoided citing their work in the present version.