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Peer Review File

Xenotransplanted Human Organoids Identify Transepithelial Zinc Transport as a Key Mediator of Intestinal Adaptation

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REVIEWER COMMENTS

Reviewer #1 (Remarks to the Author):

The authors characterize changes in human intestinal epithelial tissue in response to short gut syndrome. They use 2 human model systems to do so: hiPSC derived intestinal organoids transplanted into mice undergoing small bowel resection vs sham surgery, and specimens from human patients with clinical short gut syndrome. This is in addition to tissue analysis from murine samples of sham vs SBR mice. Through these analyses, the authors conclude a significant role for zinc metabolism in driving the adaptation response. The suggestion for use of zinc in therapy for SGS is novel, and the following revisions are recommended to better support this claim.

The use of hiPSC derived intestinal organoids transplanted into the mesentery of mice is unique. These cells are harvested and analyzed using single cell RNA sequencing. This aspect of the paper would be strengthened by the following:

1. Representative immunohistochemistry of recovered human organoids, including ZIP4 and ZIP 5 staining would be helpful.
2. The authors note cell populations of meyeloid origin. Details of events occurring within this cell population would be of value.
3. It is not clear why the authors chose to combine enterocyte cluster 1 and stem-like fibroblast clusters in their analysis, rather than comparing all enterocyte populations sham vs SBR. This data should be submitted.
4. Of note, hiPSC organoids are not in contact with the intestinal lumen of the mouse, suggesting circulating/humoral factors drive the observed changes in hiPSCs. This should be addressed in the discussion.

The authors go on to argue for the potential therapeutic benefit of zinc for SGS based on in vitro murine organoid cultures supplemented with zinc, or conversely, with zinc metabolism hindered by PTEN. They also provide zinc enriched vs zinc deplete diets to mice undergoing small bowel resection, and perform IHC of specimens from human patients with short gut syndrome.

1. Repeating the in vitro experiment using human intestinal organoids would strengthen the

translational merit of their findings.
2. The reported data for survival and weight gain in sham vs SBR mice receiving zinc supplementation did not reach statistical significance, significantly limiting the translational merit of these findings. A clear distinction between structural adaptation and functional adaptation needs to be made.
3. In human samples (recognizing the limited sample size) is there a correlation between ZIP4/ZIP5 activity and weaning from TPN? Or in zinc serum levels?

Using pathway analysis, the authors identify KLF5 as being a positive regulator of observed changes in zinc metabolism.
1. Methodology for how the authors narrowed down to KLF5 from 17 candidate transcription factors is unclear and should be expounded.
2. Bowel resection studies in mice lacking intestinal Klf5 are needed to validate this claim.
3. Similarly, KLF5 expression in human biopsy samples should be analyzed and reported.

Finally, within the text there are several opportunities for reporting fold changes of critical findings (rather than just the significance), for example: RT-PCR of intestinal epithelium (fig 3g-h), SLC39A5 in human biopsy samples (fig 5c). Presenting this data in the text will help the reader understand the strength of evidence.

Reviewer #2 (Remarks to the Author):

In this paper Sampah et al provide evidence that zinc may have previously unappreciated role being a rate limiting factor in the process of intestinal adaptation. I have several concerns regarding the experiments and conclusions.

My main concern is that the only direct in vivo evidence for zink promoting intestinal adaptation is based on experiments using SBS mice which received zink supplementation, regular diet or zinc-depleted diet. In these experiments zink was provided as zinc acetate, while acetate itself as short chain fatty acid may affect enterocyte energy metabolism and has been previously associated with intestinal adaptation. In addition, in these experiments only villus height was addressed without further insight to mucosal changes such as information of enterocyte proliferation or expression of the key transporters studied with

organoids (SLC39A4 and SLC39A5). Further, the number of animals used and proper significance levels for comparisons between the groups should be provided. Thus, more robust in vivo evidence is needed by removing/controlling the effect of acetate, by providing more detailed evidence of mucosal adaptation, and by addressing the key transporters. Relating to this, it should be specified in what chemical form was zink (as acetate?) supplemented to organoids?

In clinical part of the study, the featured immunohistology images may (unsurprisingly) suggest zink deficiency in enterocytes but distinctiveness of localization pattern remains based on 2 biopsies. The unincreased expression of SLC39A4 differs from the organoid experiments – why so? It is unclear how well the intestinal biopsy sites (jejunum vs ileum) were standardized.

There is nothing mentioned about possible limitations of the study in discussion. One inherent limitation of the organoid model is that when implanted to mesentery they are devoid of intraluminal nutrients and signaling molecules which have an essential role promoting intestinal adaptation in vivo.

In order to address the comments raised, we have performed multiple additional experiments, including key experiments to evaluate the potential role of myeloid cells in the SBS model as raised by Reviewer #1, and additional experiments to exclude the effect of acetate on intestinal adaptation as raised by Reviewer #2, with extensive modifications to the corresponding text. It is our hope that by addressing the reviewers' concerns that the work is now suitable for publication in *Nature Communications.*

Please find below a point-by-point response to the reviewers' concerns.

Reviewer #1 (Remarks to the Author):

The authors characterize changes in human intestinal epithelial tissue in response to short gut syndrome. They use 2 human model systems to do so: hiPSC derived intestinal organoids transplanted into mice undergoing small bowel resection vs sham surgery, and specimens from human patients with clinical short gut syndrome. This is in addition to tissue analysis from murine samples of sham vs SBR mice. Through these analyses, the authors conclude a significant role for zinc metabolism in driving the adaptation response. The suggestion for use of zinc in therapy for SGS is novel, and the following revisions are recommended to better support this claim.

The use of hiPSC derived intestinal organoids transplanted into the mesentery of mice is unique. These cells are harvested and analyzed using single cell RNA sequencing. This aspect of the paper would be strengthened by the following:

1. Representative immunohistochemistry of recovered human organoids, including ZIP4 and ZIP 5 staining would be helpful.

> We appreciate this point although there are barriers that make staining of ZIP4 and ZIP5 in recovered human organoids impractical. Specifically, recovered human organoids are extremely tiny, and we have prioritized the performance of single cell RNA sequencing qRT-PCR studies, which yielded the ZIP4 and ZIP5 findings. Performing immunohistochemistry on the recovered human organoids would thus require repeating all the experiments. As a means to address the Reviewer's suggestion of assessing ZIP4 and ZIP5 expression in human tissue, we point out that we examined both ZIP4 and ZIP5 expression in intestinal biopsies taken from patients. Specifically, we refer the Reviewer to Figure 6 which shows the results of the ZIP4 and ZIP5 immunohistochemistry staining in human intestinal biopsies. We hope that the Reviewer will agree that this approach provided a direct and clinically relevant assessment of ZIP4 and ZIP5 expression in human tissues, complementing our findings from the organoid model.

2. The authors note cell populations of myeloid origin. Details of events occurring within this cell population would be of value.

Thank you for this comment. In response, we have now performed an analysis of the events within cell populations of myeloid origin and note that in the SBS group, genes and pathways associated with the maintenance of intestinal stem cells, anti-inflammatory responses, and barrier function. These studies revealed that ANXA1, SOX9, and HIF1A, were upregulated in human mesenchymal cells of myeloid origin that were harvested after implantation. We have thus revised the manuscript to include a description of these findings as follows:

> *"In our study, mesenchymal cells were proportionally distributed across organoid implants derived from both SBS and sham-treated groups. However, further analysis revealed significant differences between the groups. Mesenchymal cells from the SBS group exhibited enhanced expression of genes and pathways crucial for intestinal stem cell support, anti-inflammatory responses, and barrier integrity. Notably, genes such as ANXA1, SOX9, and HIF1A were upregulated in mesenchymal cells extracted from SBS mice post-implantation. This suggests a unique adaptive response in the mesenchymal compartment."*

This information appears in the **Revised Results page 8**.

3. It is not clear why the authors chose to combine enterocyte cluster 1 and stem-like fibroblast clusters in their analysis, rather than comparing all enterocyte populations sham vs SBR. This data should be submitted.

To clarify, we compared each lineage cell type separately, comparing sham versus SBS conditions. This is why we did not combine enterocyte cluster 1 and stem-like fibroblast clusters in our analysis; instead, we compared each cluster individually, and so to clarify, the data requested by the Reviewer **had been submitted** originally.

In order to provide additional clarity, we have modified the text in the **Results, Page 7** as follows:

"For a comprehensive analysis of the key pathways involved in the adaptation process of human SBS, we conducted differential expression analysis and gene set enrichment analysis (GSEA) focusing on intestinal cells. Specifically, we compared cells from each cluster dominated by SBS-induced cells (enterocyte cluster 1 and stem-like fibroblast clusters) with corresponding cells of the same lineage representing the baseline or sham state. Hence cells from enterocyte cluster 1 (95% SBS) were compared to enterocyte 2 (59% SBS) and those from stem-like fibroblast (96% SBS) were compared to mature fibroblast (52% SBS)."

We hope this clears up this point of apparent confusion.

4. Of note, hiPSC organoids are not in contact with the intestinal lumen of the mouse, suggesting circulating/humoral factors drive the observed changes in hiPSCs. This should be addressed in the discussion.

> We appreciate this point, which we have now addressed and included in the **Revised Discussion Page 19:**
>
> "*An important consideration in our study is that the hiPSC-derived organoids were not in direct contact with the intestinal lumen of the mouse. This isolation from the lumen suggests that the observed changes in the hiPSC derived intestinal organoids are likely driven by circulating or humoral factors derived from mesenteric circulation of the host. Cytokines, growth factors, and hormones significantly influence the behavior and differentiation of intestinal stem cells. Hence, our data may not have captured the physiological and cellular effects induced by direct exposure to intestinal contents, including nutrients, microbiota, and luminal secretions, along with their associated mechanisms.*"

The authors go on to argue for the potential therapeutic benefit of zinc for SGS based on in vitro murine organoid cultures supplemented with zinc, or conversely, with zinc metabolism hindered by PTEN. They also provide zinc enriched vs zinc deplete diets to mice undergoing small bowel resection and perform IHC of specimens from human patients with short gut syndrome.

1. Repeating the in vitro experiment using human intestinal organoids would strengthen the translational merit of their findings.

> We agree and have now **included this new data** in our revised manuscript to **Revised Figure 4** (to complement the original figure 4).

2. The reported data for survival and weight gain in sham vs SBR mice receiving zinc supplementation did not reach statistical significance, significantly limiting the translational merit of these findings. A clear distinction between structural adaptation and functional adaptation needs to be made.

> The Reviewer is, respectfully, mistaken, as the reported data for weight gain did indeed reach significance between SBS mice fed with no zinc and those fed with increasing amounts of zinc (see Figure 5a). As stated in the Results (page 12): "Strikingly, we discovered that SBS mice receiving zinc supplementation exhibited significant weight gain compared to those on standard or zinc-depleted diets (Fig. 5). By day 7, mice on the standard diet lost 13.38 ± 1.11% ($p<0.05$), whereas mice in the zinc supplementation group had lost approximately 7.1 ± 1.19% of their original weight. Furthermore, while differences in the survival curves did not reach statistical significance, zinc supplementation showed a trend

toward improved survival rates in SBS mice compared to control SBS mice at day 7, with survival rates of 85.7% versus 66.67%, respectively (Fig. 5b)."

In order to add further clarity to this finding, we have now **added an additional figure** to the supplementary data (**supplementary fig. 2**), showing weight gain and survival data for sham and SBS mice across different diet groups. This data is described in the **Revised Results page 12**, and reveals improved weight gain for SBS mice receiving supplementation with zinc, but not for zinc supplemented sham mice. We hope that this additional data helps to clarify the data regarding the protective role of Zn in weight loss and survival in SBS mice.

Further, we appreciate the comment regarding the need to make the distinction between structural adaptation and functional adaptation, and have done just that. We thus point the Reviewer to the **Revised text pages 11-12** which reveals that the morphometric data represents structural adaptation, while functional adaptation is revealed by the growth velocity curves, proliferation and enzyme expression – all of which are improved by Zn in the setting of SBS. Specifically, as stated on page 11-12:

> "*Functional adaptation was indirectly assessed by monitoring changes in body weight during the post-operative period. We assessed structural adaptation histologically by measuring villus height and crypt depth and compared these parameters across the various treatment groups. We also evaluated functional adaptation directly by conducting immunohistological analyses. This involved the use of antibodies against proliferative markers such as BRDU, Ki67, and PCNA, coupled with measurements of sucrase-isomaltase enzyme levels, to assess the regenerative and digestive capabilities across the treated groups."*

3. In human samples (recognizing the limited sample size) is there a correlation between ZIP4/ZIP5 activity and weaning from TPN? Or in zinc serum levels?

We appreciate this question. However, due to the limited sample size and incomplete electronic medical record (EMR) data, we are currently unable to establish such correlations in this study. That said, based on the current findings, we are in the early stages of a retrospective clinical study of SBS patients which aims to determine the presence of clinical correlations concerning ZIP4/ZIP5 activity, zinc levels, and TPN dependency. This investigation is of course beyond the scope of the current manuscript, but we recognize its significance and are committed to addressing it in subsequent studies.

Using pathway analysis, the authors identify KLF5 as being a positive regulator of observed changes in zinc metabolism.

1. Methodology for how the authors narrowed down to KLF5 from 17 candidate transcription factors is unclear and should be expounded.

We appreciate the suggestion to provide more clarity on the methodology used to narrow down to KLF5 from the 17 candidate transcription factors. We have modified the manuscript on the **Revised Results, pages 14-15** as follows:

"*To identify the key transcription factors governing the expression of ZIP4 and ZIP5 in short bowel syndrome (SBS), we employed a combination of bioinformatics analyses and experimental validation. We determined the transcriptional regulatory network governing the expression of ZIP4 and ZIP5 in short bowel syndrome (SBS), by employing the pySCENIC tool to infer transcription factor activity based on gene expression patterns and known transcription factor binding site information (**Fig. 7a**). Through this analysis, we identified 17 candidate transcription factors that potentially upregulate the regulon encompassing SLC39A4 and SLC39A5.*

*To narrow down these transcription factors, we identified genes with the highest co-expression to SLC39A4 and SLC39A5, creating a potential regulon. We then correlated the expression of all these genes to all 17 transcription factors and found that four transcription factors stood out as having a strong correlation across this potential regulon: KLF5, ESSRA, HNF4A, and HNF4G (**Fig. 7b**). To further examine these potential regulators, we looked at their expression in SBS compared to sham-derived cells and found that all four had significantly higher expression in SBS cells (**Fig 7c**). We next evaluated the Area Under the Curve (AUC) scores generated by pySCENIC, which are used as a metric of regulon enrichment. This value also indicates that the regulons of these four transcription factors are significantly enriched in SBS compared to SHAM (**Fig 7d**). Since the expression of SLC39A4 and SLC39A5 are specific to enterocytes, we checked our pool of potential regulators for this pattern. Among these candidate transcription factors, it became evident that ESSRA was not exclusively expressed in enterocyte cells; its expression was also observed in various other clusters. When considering the remaining candidates, KLF5 had the highest expression across enterocytes, suggesting it as the primary regulator responsible for the transcription of ZIP4, ZIP5, and their associated proteins within the identified regulon in enterocyte cells (**Fig. 7e**). Finally, we validated the expression and activity of Klf5 in our experimental model. Our analysis revealed a noteworthy upregulation of Klf5 in native mouse SBS enterocytes in comparison to sham controls (**Fig. 7f**). Most remarkably, though overall levels were low compared to housekeeping genes, KLF5 expression was statistically higher in tissue obtained from SBS patient biopsies compared to control patient intestinal tissue (**Fig. 7g**).*"

2. Bowel resection studies in mice lacking intestinal Klf5 are needed to validate this claim.

We agree that it would be nice to perform bowel resection studies in mice lacking intestinal *Klf5* would be valuable to validate our findings. However, we would like to point out that complete knockout of *Klf5* is embryonic lethal, which precludes its use for postnatal studies. While an inducible intestine-specific deletion of *Klf5* mouse strain exists (Villin-Cre-ER^{T2}; Klf5$^{fl/f}$) this strain presents significant limitations due to the poor phenotype observed upon tamoxifen treatment. Specifically, these mice exhibit marked epithelial disruption, making them unsuitable for studying the SBS model as their compromised intestinal integrity would confound the results. (Nandan et al. Inducible intestine-specific deletion of Krüppel-like factor 5 is characterized by a regenerative response in adult mouse colon. Dev Biol. 2014 Mar 15;387(2):191-202.). We therefore respectfully hope that the reviewer will agree that while conducting knockout experiments to study the role of KLF5 in SBS would be of benefit, such studies are not practical at this time. We do also point out that the current studies focus on the demonstration of the zinc pathways as therapeutic targets, as demonstrated by our ability to reverse weight loss in clinically relevant mouse models of disease through zinc supplementation in the diet.

3. Similarly, KLF5 expression in human biopsy samples should be analyzed and reported.

We thank the reviewer for this suggestion. In response, we have **now performed** RT-qPCR on human biopsy samples using primers for *KLF5*, and our results show that *KLF5* expression is significantly upregulated in SBS patient intestinal tissue compared to controls. This data is included in **Revised Results fig. 6** and in the **Revised Discussion page 15** as follows:

> *"Most remarkably, though overall levels were low compared to housekeeping genes, KLF5 expression was statistically higher in tissue obtained from SBS patient biopsies compared to control patient intestinal tissue."*

Finally, within the text there are several opportunities for reporting fold changes of critical findings (rather than just the significance), for example: RT-PCR of intestinal epithelium (fig 3g-h), SLC39A5 in human biopsy samples (fig 5c).

Presenting this data in the text will help the reader understand the strength of evidence.

We appreciate this comment and have now added fold changes in RT-PCR in the figure legends of figures 3g-f, 6b-c and 7f-g and described these in the results section.

<u>Reviewer #2 (Remarks to the Author):</u>

In this paper Sampah et al provide evidence that zinc may have previously unappreciated role being a rate limiting factor in the process of intestinal adaptation. I have several concerns regarding the experiments and conclusions.

My main concern is that the only direct in vivo evidence for zink promoting intestinal adaptation is based on experiments using SBS mice which received zink supplementation, regular diet or zinc-depleted diet.

In these experiments zink was provided as zinc acetate, while acetate itself as short chain fatty acid may affect enterocyte energy metabolism and has been previously associated with intestinal adaptation.

> We thank the reviewer for raising the issue of the potential confounding effect of acetate itself on intestinal adaptation in our SBS mouse model. To address the possibility that acetate could affect intestinal adaptation, we have **now performed additional experiments** in which sodium acetate was administered to mice that had undergone the SBS model. As shown in **the new Supplementary Figure 2 (f, h-j)**, growth curves and morphometric analysis of sodium acetate treated mice with SBS showed no evidence of enhanced intestinal adaptation with acetate alone, findings that were completely different from the effects of zinc acetate. These findings exclude a role for acetate, and implicate zinc as the causative agent in the adaptation response. These findings are also summarized in the **revised Results pages 12 and 13**.

In addition, in these experiments only villus height was addressed without further insight to mucosal changes such as information of enterocyte proliferation or expression of the key transporters studied with organoids (SLC39A4 and SLC39A5).

> In response to this comment, we have now **performed additional experiments** in order to provide data on these additional endpoints. Specifically, we have performed qRT-PCR for Ki67 and PCNA on intestinal tissue for these mice and completed immunostaining for Ki67, BRDU and PCNA to assess enterocyte proliferation. These data are shown in **Revised Figure 5** reveal that Ki67 expression is increased at the RNA level in SBS mice treated with zinc. By immunostaining, zinc treated SBS mice showed increase in proliferation markers BRDU, Ki67, and PCNA, as well as SI. The findings are summarized on **page 13 of the revised results**.
>
> Concerning zinc transporter expression, we respectfully draw the Reviewer's attention to current **Figure 3** using intestinal tissue from mice qRT-pCR and western blots, which confirms the increased Slc39a4/Zip4 and Slc39a5/Zip5 expression in the mucosa of SBS mice.

Further, the number of animals used and proper significance levels for comparisons between the groups should be provided.

> We have **now provided** in the results and figure legends detailed information on the number of animals used in each experimental group and the statistical significance levels for comparisons between groups in the revised manuscript.

Thus, more robust in vivo evidence is needed by removing/controlling the effect of acetate, by providing more detailed evidence of mucosal adaptation, and by addressing the key transporters.

> We have now addressed these concerns as indicated above.

Relating to this, it should be specified in what chemical form was zink (as acetate?) supplemented to organoids?

> In the mouse enteroid data presented in **figure 4 a - m**, zinc was supplemented as an acetate compound. We have now specified that Zinc was provided as Zinc acetate in the **Revised Results (pages 10-11**).

In clinical part of the study, the featured immunohistology images may (unsurprisingly) suggest zink deficiency in enterocytes but distinctiveness of localization pattern remains based on 2 biopsies.

> Thank you for your observations regarding the immunohistology images. The Revised text on **page 50** now indicates that we have analyzed **over 25** biopsies from participants across both the SBS group and the control group and have included representative examples from one patient in each group.

The unincreased expression of SLC39A4 differs from the organoid experiments – why so?

> The lack of increased expression of SLC39A4 between SBS and control biopsies is indeed intriguing. As discussed in our manuscript on **pages14 and 18**, although SLC39A4 mRNA levels did not differ significantly, we observed a notable difference at the protein level. Specifically, ZIP4 protein was predominantly localized at the cell surface in enterocytes from SBS patients, compared to a mainly cytoplasmic presence in controls. This suggests an upregulation of zinc transport mechanisms at the protein level, rather than at the mRNA expression level, potentially due to post-transcriptional modifications or altered protein trafficking in response to the zinc deficiency in SBS patients. This discrepancy underscores the complexity of zinc regulation and highlights the importance of considering multiple levels of gene expression regulation in such studies.

It is unclear how well the intestinal biopsy sites (jejunum vs ileum) were standardized.

In this study, biopsy sites were dependent upon the surgical indications specific to each patient. To ensure consistency in data analysis, we referenced each patient's operative summary to determine the location of the biopsies within the intestinal tract. This approach allowed us to correlate the histological findings with the relevant segment of intestine.

There is nothing mentioned about possible limitations of the study in discussion. One inherent limitation of the organoid model is that when implanted to mesentery they are devoid of intraluminal nutrients and signaling molecules which have an essential role promoting intestinal adaptation in vivo.

We agree. We have added the following to **the Revised discussion section on page 19:**

"An important consideration in our study is that the hiPSC-derived organoids were not in direct contact with the intestinal lumen of the mouse. This isolation from the lumen suggests that the observed changes in the hiPSC derived intestinal organoids are likely driven by circulating or humoral factors derived from mesenteric circulation of the host. Cytokines, growth factors, and hormones significantly influence the behavior and differentiation of intestinal stem cells. Hence, our data may not have captured the physiological and cellular effects induced by direct exposure to intestinal contents, including nutrients, microbiota, and luminal secretions, along with their associated mechanisms."

REVIEWERS' COMMENTS

Reviewer #1 (Remarks to the Author):

The authors have done a nice job responding to reviewer concerns. The paper reads easily and makes a clear and convincing argument for zinc deficiency/supplementation in SBS.

For clinicians, a brief discussion of baseline dietary zinc requirements (per kg? does it vary by age, etc), primary dietary sources of zinc, typical absorptive location (ie proximal vs distal bowel), and bodily storage/reservoirs would be nice, but is not mandatory for publication.

Minor typographical point in line 134 (should be 75% of small bowel, not ileum?)

Thank you for the opportunity to review this interesting and clinically relevant study.

Reviewer #2 (Remarks to the Author):

Thank you for prompt and comprehensive response to my suggestions. I have no further conecrns.

Please find below a point-by-point response to the reviewers' concerns.

Reviewer #1 (Remarks to the Author):

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The authors have done a nice job responding to reviewer concerns. The paper reads easily and makes a clear and convincing argument for zinc deficiency/supplementation in SBS.

For clinicians, a brief discussion of baseline dietary zinc requirements (per kg? does it vary by age, etc), primary dietary sources of zinc, typical absorptive location (ie proximal vs distal bowel), and bodily storage/reservoirs would be nice, but is not mandatory for publication.

> We agree that this additional discussion would enhance the impact of our manuscript. In response to your suggestion, we have added the following paragraph to the discussion section of the manuscript:
>
> > "*Zinc needs vary by age and physiological status, with daily recommendations ranging from 2 mg for infants to 11 mg for adults. In healthy individuals, zinc absorption primarily occurs in the proximal small intestine. Various factors can inhibit this process, including phytates, dietary fiber, excessive calcium and iron levels, oxalates, polyphenols, certain medications, and excessive alcohol consumption. These inhibitors can bind to zinc, form insoluble complexes, or compete for absorption, reducing zinc's bioavailability. The body stores zinc mainly in skeletal muscle and bone, with additional storage in the liver, skin, kidneys, and prostate. The body stores zinc mainly in skeletal muscle and bone, with additional storage in the liver, skin, kidneys, and prostate. Whole-body zinc content remains stable over a wide range of dietary zinc concentrations due to efficient homeostatic mechanisms. Excess endogenous zinc is secreted into the intestine and excreted in feces.*"

Minor typographical point in line 134 (should be 75% of small bowel, not ileum?)

> We have corrected this error.

Thank you for the opportunity to review this interesting and clinically relevant study.