

## PEER REVIEW HISTORY

BMJ Paediatrics Open publishes all reviews undertaken for accepted manuscripts. Reviewers are asked to complete a checklist review form and are provided with free text boxes to elaborate on their assessment. These free text comments are reproduced below.

### ARTICLE DETAILS

<b>TITLE (PROVISIONAL)</b>	Copper and zinc concentrations in the breast milk of mothers undergoing treatment for Wilson's disease: a prospective study
<b>AUTHORS</b>	Kodama, Hiroko Anan, Yasumi Izumi, Yoichi Sato, Yasuhiro Ogra, Yasumitsu

### VERSION 1 – REVIEW

<b>REVIEWER</b>	Reviewer name: Prof. Maria C Linder Institution and Country: California State University Fullerton, United States Competing interests: None
<b>REVIEW RETURNED</b>	11-Jan-2021

<b>GENERAL COMMENTS</b>	<p>In this study, the authors investigated whether they could detect trientine or penicillamine chelators or excess Zn in the milk of mothers under long term treatment for Wilson disease, and also whether Cu and Zn levels in colostrum or milk were abnormal. Using mass spectrometry and size exclusion HPLC they failed to detect Cu-trientine or Cu-penicillamine, and total levels of Cu and Zn in the milk were generally normal. This is useful information. Some blood serum levels of Cu and Zn were also measured, and showed some elevation in serum Zn (though not in milk) in those WD subjects taking Zn treatment.</p> <p>The only problem with the data provided have to do with the identification of components in the milk that bind copper. The investigators are using a gel chromatography procedure from 1981, and keep referring to that paper for proving what they say about the peaks shown in the chromatographs. Although they may be correct, lots has changed in 1981, and even if they were using the same columns and buffer conditions, one would have to see confirmation of what they identify as Cu-lactalbumin, and prove that one of the peaks shown is indeed not ceruloplasmin (as they did to prove their inability to detect the Cu-trientine or Cu-penicillamine complexes – by showing where they would elute in the HPLC system they are using) if they want to draw valid conclusions about these matters. Thus, unless such information is added, this part of the manuscript shod be deleted.</p> <p>There are also some minor issues: In the Introduction and some other places, the authors fail to add references in support of their statements, or in some cases add the references way at the end of sentences in which they make specific claims. For example in the first Paragraph of the Introduction, each statement should have a specific reference added to it, which supports the statement. On the second to last page of the Discussion, lines 20-22 state that penicillamine was bound to “the plasma protein” (ref. 22). What protein is that? On the same page, the first statement in the next paragraph needs a reference (that WD babies undergo normal development).</p>
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	<p>On the first page of the Introduction, line 15, it says that ceruloplasmin accounts for 90% of serum copper – which has been floating around for years, but is not correct. It's more like 50-70%. In Methods, describing the copper distribution profiles, line 19, the quantities of the Cu-complexes applied to the HPLC-ICP-MS system (in the 20 uL) need to be listed.</p> <p>Legends to figures (or tables) should not include interpretation (which should be only in the text) – as in the case of figure 1, the last two sentences.</p> <p>Second to last page of the Discussion, line 15, it is stated that “ATP7A acts as a copper absorbent”. That is not a correct statement. Lines 19-22: It is stated that “ATP7B transports copper from hepatocytes to the blood and bile ducts”. It should be made clear that it secretes copper into the blood in the form of ceruloplasmin, Currently, the statement suggests it releases ionic copper into the blood (which is something that ATP7A does, not ATP7B).</p> <p>Line 24 should include mammary gland as a tissue that expresses both ATP7B and ATP7A.</p> <p>Line 29: This sentence is not correct (ATP7A does not regulate cellular efflux of copper into milk). Moreover, the reference listed (22) has nothing to do with this topic.</p> <p>Lines 33-36: the “dual role” for ATP7A that is referred to here should be explained to the reader – including the evidence.</p>
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<b>REVIEWER</b>	<p>Reviewer name: Dr. Facundo Garcia-Bournissen  Institution and Country: London Health Sciences Centre, Canada  Competing interests: None</p>
<b>REVIEW RETURNED</b>	24-Jan-2021

<b>GENERAL COMMENTS</b>	<p>This is the largest (and maybe only) cohort of Wilson disease lactating women treated with either penicillamine, trintene or zinc, for whom there is data on copper and zinc (and penicillamine or trintene) breastmilk concentrations, showing that they are within normal limits. These data support, to the extent possible, that breastfeeding is possible for Wilson disease patients under treatment.</p> <p>The manuscript has small issues, but I believe that it is interesting and clinically useful.</p> <p>Minor comments:</p> <ul style="list-style-type: none"> <li>- Title: It sounds a bit too prescriptive. I understand the intention to attract attention to the manuscript, but I think it should be a bit more careful (e.g. “Low transfer of copper into breastmilk suggests that mothers with Wilson disease under treatment may safely breastfeed”)</li> <li>- Abstract: The objectives section should include only the objectives (e.g. “clarified whether they could safely breastfeed their infants” would probably be a conclusion)</li> <li>- Abstract: The phrase “Breast milk samples were analyzed at the institute of affiliation (3) in Chiba” is not clear. What does the “(3)” mean?</li> <li>- Abstract: Based on the description of the participants, this study seems like a prospective cohort study (e.g. this should be the description provided in “design”), or at least a prospective case series</li> <li>- Conclusions: The conclusion in the abstract does not address whether trientine, penicillamine cross into breastmilk (just the copper and zinc levels), which is in fact described in the manuscript. I would add this information to the abstract (understanding that the abstract is the only part that many people would read...) – concluding that mothers under treatment can breastfeed safely is a very strong conclusion for their limited data. Maybe they should state that breastmilk from mothers with Wilson disease under treatment with trentine, penicillamine or zinc had normal copper and zinc levels, and therefore breastfed babies would not be expected to be exposed to potentially toxic levels of these metals given the</li> </ul>
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	<p>limited data presented in the manuscript.</p> <p>- In the "what is known about the subject?" The authors state that "no study has evaluated the safety of breastfeeding by mothers undergoing treatment for WD". This does not seem completely correct. Even though the data is limited, there are a few published studies in abstract form (only one of these is mentioned in the references – all should probably be quoted, even if 2 of them are abstracts from a meeting, from one of the authors of this paper – other authors of the abstracts are not part of this paper):</p> <ul style="list-style-type: none"> <li>o Shiga K, Kaga H, Kodama H et al. Copper and zinc concentrations in the breast milk of mothers with Wilson disease and effects on infants. <i>J Inherit Metab Dis</i>. 2006;29 (Suppl 1):139. Abstract. DOI: 10.1007/s10545-006-9995-6.</li> <li>o Izumi Y. [Can mothers with Wilson's disease give her breast milk to their infant?]. <i>Teikyo Med J</i>. 2012;35:17-24.</li> <li>o Kaga F, Kodama H, Siga K et al. Copper and zinc status in the breast milk of mothers with Wilson disease. <i>J Inherit Metab Dis</i> 2008;31 (Suppl 1):157. Abstract. DOI: 10.1007/s10545-008-9975-0.</li> </ul>
<b>REVIEWER</b>	<p>Reviewer name: Dr. Peter Flom  Institution and Country: Peter Flom Consulting, United States  Competing interests: None</p>
<b>REVIEW RETURNED</b>	15-Jan-2021
<b>GENERAL COMMENTS</b>	<p>I confine my remarks to statistical aspects of this paper.</p> <p>These are appropriately done and I recommend publication</p>

### VERSION 1 – AUTHOR RESPONSE

#### Response to reviewers

##### Reviewer: 1

The only problem with the data provided have to do with the identification of components in the milk that bind copper. The investigators are using a gel chromatography procedure from 1981, and keep referring to that paper for proving what they say about the peaks shown in the chromatographs. Although they may be correct, lots has changed in 1981, and even if they were using the same columns and buffer conditions, one would have to see confirmation of what they identify as Cu-lactalbumin, and prove that one of the peaks shown is indeed not ceruloplasmin (as they did to prove their inability to detect the Cu-trientine or Cu-penicillamine complexes – by showing where they would elute in the HPLC system they are using) if they want to draw valid conclusions about these matters. Thus, unless such information is added, this part of the manuscript shod be deleted.

Response: Thank you for your comments. Since our technique was validated, we believe that our results are in line with those reported in previous findings. In addition, previous studies have shown that lactalbumin is a major milk protein that binds to Cu (Hoac T et al. Separation of Selenium, Zinc and Copper compounds in the bovine whey using size exlusion chromatography linked to inductively coupled plasma mass spectrometry. *Agricultural Food Chemistry* 2007; 55: 4237-4243). However, in this study, we did not perform the solid identification of ceruloplasmin. Therefore, as per your suggestion, the mention of ceruloplasmin has been removed from the revised manuscript.

##### There are also some minor issues:

In the Introduction and some other places, the authors fail to add references in support of their statements, or in some cases add the references way at the end of sentences in which they make specific claims. For example in the first Paragraph of the Introduction, each statement should have a specific reference added to it, which supports the statement.

Response: Thank you for your comment. We have cited the relevant references throughout the manuscript, for accuracy, as per the Reviewer's suggestion.

On the second to last page of the Discussion, lines 20-22 state that penicillamine was bound to "the plasma protein" (ref. 22). What protein is that?

Response: Thank you for your question. The plasma protein may be albumin, in line with the data included in a previously published study (ref). However, since we are not sure, we decided not to mention any specific protein in the revised manuscript that reads as follows:

"Another study demonstrated that in rats administered penicillamine, nearly all penicillamine in the plasma was bound to plasma proteins. [22]"

On the same page, the first statement in the next paragraph needs a reference (that WD babies undergo normal development).

Response: Thank you for your comments. The quote "WD babies undergo normal development" refers to our results described in the last sentence of page 5 (Methods Subjects and biological samples) and hence no reference is needed.

However, we have revised the text as follows to avoid confusion:

"In addition, babies breastfed by mothers with WD undergoing treatment exhibited normal development in our study."

On the first page of the Introduction, line 15, it says that ceruloplasmin accounts for 90% of serum copper – which has been floating around for years, but is not correct. It's more like 50-70%.

Response: Thank you for your comment. We have revised the text accordingly and have included a more recent reference (2019), as follows:

"Ceruloplasmin-copper accounts for approximately 60–95% of the serum copper levels. [1,3,5]"

In Methods, describing the copper distribution profiles, line 19, the quantities of the Cu-complexes applied to the HPLC-ICP-MS system (in the 20  $\mu$ L) need to be listed.

Response: Thank you for your suggestion. We have included the concentration of the Cu-complexes in the revised manuscript, accordingly.

Legends to figures (or tables) should not include interpretation (which should be only in the text) – as in the case of figure 1, the last two sentences.

Response: Thank you for your comment. The last two sentences of Figure 1 were deleted accordingly.

Second to last page of the Discussion, line 15, it is stated that "ATP7A acts as a copper absorbent". That is not a correct statement.

Response: Thank you for your comment. We have revised the sentence, for accuracy, as follows:

"Moreover, ATP7A is found in intestinal cells, transporting copper from enterocytes into the blood."

Lines 19-22: It is stated that "ATP7B transports copper from hepatocytes to the blood and bile ducts". It should be made clear that it secretes copper into the blood in the form of ceruloplasmin. Currently, the statement suggests it releases ionic copper into the blood (which is something that ATP7A does, not ATP7B).

Response: Thank you for your comment. We have revised the text accordingly, as follows:

"ATP7B transfers copper from the cytosol to the trans-Golgi network of hepatocytes. Subsequently, copper in the trans-Golgi network is incorporated into apo-ceruloplasmin and secreted as ceruloplasmin into the blood. ATP7B also excretes copper into the bile."

Line 24 should include mammary gland as a tissue that expresses both ATP7B and ATP7A.

Response: Thank you for this important suggestion. We have included mammary glands in the list of tissue expressing both ATP7B and ATP7A:

"Both ATP7A and ATP7B are expressed in various organs, including the kidneys, brain, lungs, placenta, and mammary glands."

Line 29: This sentence is not correct (ATP7A does not regulate cellular efflux of copper into milk). Moreover, the reference listed (22) has nothing to do with this topic.

Response: Thank you for the remark. Indeed, reference 22 was incorrect and we apologize for this oversight. We have revised the sentences as follows and have included the correct reference.

"Meanwhile, in human breast cells, ATP7A may be involved in the cellular efflux of copper into milk.[26]"

26. Ackland ML, Anikijenko P, Michalczyk A, et al. Expression of menkes copper-transporting ATPase, MNK, in the lactating human breast: possible role in copper transport into milk. *J Histochem Cytochem* 1999;47:1553–62. Doi: 10.1177/002215549904701207.

Lines 33-36: the "dual role" for ATP7A that is referred to here should be explained to the reader – including the evidence.

Response: Thank you for your suggestion. We have revised the text as follows:

"ATP7A was also detected in both luminal and serosal membranes, which suggests a dual role, i.e., exporting copper into milk and importing it back into maternal circulation.[24,27]"

27. Wadwa J, Chu YH, Nguyen N, et al. Effects of ATP7A overexpression in mice on copper transport and metabolism in lactation and gestation. *Physiol Rep* 2014;2:e00195. doi: 10.1002/phy2.195.

Reviewer: 2

Thank you for all of the previous suggestions. They were essential for the improvement of our manuscript.

Reviewer: 3

Minor comments:

- Title: It sounds a bit too prescriptive. I understand the intention to attract attention to the manuscript, but I think it should be a bit more careful (e.g. "Low transfer of copper into breastmilk suggests that mothers with Wilson disease under treatment may safely breastfeed")

Response: Thank you for your suggestion. As the journal specify not to include the results of the study in the tile, and in line with your suggestion, we have revised the title as follows:

"Can mothers with Wilson disease under treatment safely breastfeed? Results of an observational prospective study."

- Abstract: The objectives section should include only the objectives (e.g. "clarified whether they could safely breastfeed their infants" would probably be a conclusion)

Response: Thank you for your comment. We have revised the objectives for accuracy, as follows:

"To evaluate the concentrations of copper and zinc in the breast milk of mothers undergoing treatment for Wilson's disease (WD) and clarify whether they can safely breastfeed their infants."

- Abstract: The phrase "Breast milk samples were analyzed at the institute of affiliation (3) in Chiba" is not clear. What does the "(3)" mean?

Response: Thank you for your question. After your mention, we realized that the mention of number (3) was unclear. Therefore, we have updated the manuscript, for accuracy, as follows:

"Breast milk samples were analyzed at Chiba University."

- Abstract: Based on the description of the participants, this study seems like a prospective cohort study (e.g. this should be the description provided in "design"), or at least a prospective case series

Response: Thank you for your comment. Indeed, this was a prospective study. Therefore, the Design subsection of the Abstract has been revised as follows:

"This study was an observational and prospective study in an individual-based case series.

- Conclusions: The conclusion in the abstract does not address whether trientine, penicillamine cross into breastmilk (just the copper and zinc levels), which is in fact described in the manuscript. I would add this information to the abstract (understanding that the abstract is the only part that many people would read...)

Response: Thank you for your comment. Of note, we have indicated in the manuscript that "No peak was detected for trientine- or penicillamine-bound copper". In fact, in this study, we did not determine the concentrations of trientine and penicillamine; therefore, we neither believe this should be included in the abstract nor that the final conclusions require further revision.

- concluding that mothers under treatment can breastfeed safely is a very strong conclusion for their limited data. Maybe they should state that breastmilk from mothers with Wilson disease under treatment with trientine, penicillamine or zinc had normal copper and zinc levels, and therefore breastfed babies would not be expected to be exposed to potentially toxic levels of these metals given the limited data presented in the manuscript.

Response: Thank you for your comments. The last sentence of the Conclusions states that "These results suggest that mothers undergoing treatment for WD can safely breastfeed their infants". Of note, in this sentence we used "suggest" rather than "indicate" or "clearly show" for the interpretational accuracy of our results.

In the "what is known about the subject?" The authors state that "no study has evaluated the safety of breastfeeding by mothers undergoing treatment for WD". This does not seem completely correct. Even though the data is limited, there are a few published studies in abstract form (only one of these is mentioned in the references – all should probably be quoted, even if 2 of them are abstracts from a meeting, from one of the authors of this paper – other authors of the abstracts are not part of this paper):

o Shiga K, Kaga H, Kodama H et al. Copper and zinc concentrations in the breast milk of mothers with Wilson disease and effects on infants. *J Inherit Metab Dis*. 2006;29 (Suppl 1):139. Abstract. DOI: 10.1007/s10545-006-9995-6.

o Izumi Y. [Can mothers with Wilson's disease give her breast milk to their infant?]. *Teikyo Med J*. 2012;35:17-24.

o Kaga F, Kodama H, Siga K et al. Copper and zinc status in the breast milk of mothers with Wilson disease. *J Inherit Metab Dis* 2008;31 (Suppl 1):157. Abstract. DOI: 10.1007/s10545-008-9975-0.

Response: Thank you for your comments and suggestions. In fact, the suggested papers (Shiga, izumi, Kaga) were published by our research group. The Izumi's paper was cited in this study as reference n<sup>o</sup> 18, while the studies by papers Shiga and Kaga refer to abstracts presented at International Meetings, with data parallel to that in the Izumi's study. Nevertheless, for accuracy, we have revised the statement in the "What is known about the subject" as follows:

"Meanwhile, the safety of breastfeeding by mothers undergoing treatment for WD has been evaluated by a limited number of studies."

### VERSION 2 – REVIEW

<b>REVIEWER</b>	Reviewer name: Prof. Maria C Linder Institution and Country: California State University Fullerton, United States Competing interests: None
<b>REVIEW RETURNED</b>	31-Mar-2021

<b>GENERAL COMMENTS</b>	<p>I was unable to find a point-by-point response to reviewer comments, which required much more work on my part. Most of my concerns have been addressed. However, one persists: For the statement in the Introduction about copper in blood plasma attributable to ceruloplasmin, the authors modified "90%" to "60-95%", but without providing the appropriate references for the lower range of values. Information on this can be found in my most recent reviews of the evidence against the 90-95% values (so common in the literature) and are the following:</p> <p>Linder, M.C. (2021) Apoceruloplasmin: Abundance, detection, formation, and metabolism. <i>Biomedicines</i> 9:233. <a href="https://doi.org/10.3390/biomedicines9030233">https://doi.org/10.3390/biomedicines9030233</a></p> <p>Linder, M.C. (2016) Ceruloplasmin and other copper binding components of blood plasma and their functions: An update. <i>Metallomics</i> 8: 887-905.</p> <p>I would hope the authors would read the evidence and consider whether to further revise their statement about ceruloplasmin copper levels.</p>
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<b>REVIEWER</b>	Reviewer name: Dr. Facundo Garcia-Bournissen Institution and Country: London Health Sciences Centre, Canada Competing interests: None
<b>REVIEW RETURNED</b>	31-Mar-2021

<b>GENERAL COMMENTS</b>	My previous comments have been addressed in full.
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### VERSION 2 – AUTHOR RESPONSE

#### Response to Reviewers

1) Comment: Title should amend to "Copper and zinc concentrations in the breast milk of mothers undergoing treatment for Wilson's disease: a prospective study". Titles need to reflect the study NOT the findings

Response: The title was changed to "Copper and zinc concentrations in breast milk of mothers undergoing treatment for Wilson's disease: a prospective study", as suggested.

2) Comment: Refer to concentrations NOT levels of copper and zinc throughout the paper.

Response: Copper and zinc levels were changed to indicate concentrations.

3) Comment: A limitation is that you did NOT determine concentrations of trientine or penicillamine in breast milk. This needs to be mentioned.

Response: We have added the sentence, "In addition, the concentrations of trientine or penicillamine in breast milk were not examined in this study," in lines 7-8 of page 16 (in the Discussion).

4) Comment: Add the median concentrations of copper and zinc in breast milk for patients and control to the abstract.

Response: The mean concentrations of copper and zinc in breast milk for patients and control have been added to the abstract.

5) Comment: Add the actual P values for statistical comparisons.

Response: The P values have been added in Table 2.

Statistical analysis showed that the zinc concentration in the breast milk of the mothers treated with zinc is significantly different from the zinc concentration in controls, with a p value below 0.05. Thus, we have added a new figure of these data (Figure 2). In addition, we rewrote the results in the Abstract and in the Results section as follows:

"The zinc concentration in the breast milk of patients treated with zinc was significantly higher than that in control milk (Table 2). However, as shown in Figure 2, the zinc concentration in each patient treated with zinc was below the maximum concentration in controls. This result suggests that mothers treated with zinc can safely breastfeed."

6) Comment: Methods delete "Spearman's correlation coefficient was used to investigate relevant correlations. " You have not described these

Response: The sentence has been deleted, as requested.

7) Comment: For the statement in the Introduction about copper in blood plasma attributable to ceruloplasmin, the authors modified "90%" to "60-95%", but without providing the appropriate references for the lower range of values. Information on this can be found in my most recent reviews of the evidence against the 90-95% values (so common in the literature) and are the following:

Linder, M.C. (2021) Apoceruloplasmin: Abundance, detection, formation, and metabolism. *Biomedicines* 9:233. <https://doi.org/10.3390/biomedicines9030233>

Linder, M.C. (2016) Ceruloplasmin and other copper binding components of blood plasma and their functions: An update. *Metallomics* 8: 887-905.

I would hope the authors would read the evidence and consider whether to further revise their statement about ceruloplasmin copper levels.

Response: The sentence has been rephrased and a reference has been added (Reference No 5, Linder MC, Apoceruloplasmin: Abundance, detection, formation, and metabolism. *Biomedicine* 2021; 9: 233. <https://doi.org/10.3390/biomedicines9030233>).