nature portfolio

Peer Review File

Ceruloplasmin administration in the preclinical mouse model of aceruloplasminemia reveals a sex-related variation in biodistribution

Corresponding Author: Dr Massimo Alessio

This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)
In this manuscript the authors follow up on their previous studies on the use of Cp for enzyme replacement therapy in the mouse model of aceruloplasminemia. The novelty of the present work compared to their previous CommBiol paper is the finding that there appear to be sex-related changes in the distribution of Cp administered to CpKO mice. Male and female Cp-KO mice of 6 and 10 months of age are analyzed and compared to wild type mice for distribution of 64Cu-labelled Cp evaluated by PET/CT and ex vivo. The results are very interesting and potentially relevant for design of sex-specific therapy for aceruloplasminemia patients. The small number of animals makes some of the statistics a bit tricky (e.g., significant differences in 64Cu uptake in cerebellum at 10 vs 6 months of age appear to be due to a single individual, see fig 2b,d and fig 3b,c) but trends are nevertheless visible. The discussion could be expanded by adding information (if available) on the health of the CpKO mice: are there any sex-specific differences/signs of neurodegeneration at 10 months of age?
Another point that deserves some discussion is related to the fact that the authors assume that 64Cu is a proxy for Cp. In the previous study (CommBiol 2024) Cp was measured by ELISA on ex vivo samples, is it possible to confirm the presence of the protein also in this case? In the literature there is evidence that Cp may be a source of copper for cells and that only 64Cu is taken up in cells incubated with double labelled Cp (64Cu and 125I) (some papers published around 1990 and more recently in 2016), so this should be discussed.

Minor points
1. please uniform radioactivity units: in the labeling reaction the authors indicate that 2 mCi of copper bound to Cp (0.2 mg), then it is stated that ca. 5 MBq of 64Cu-Cp are injected in the caudal vein of mice. Please indicate the amount of protein that is actually employed and how does it compare to the previous 2018 and 2024 studies.
2. in figure S1b the 'brain' panel is duplicated and the 'eyes' panel is missing

Reviewer #2

(Remarks to the Author)
The authors study a mouse model (cpKO) of aceruloplasminemia (ACP). These mice develop symptoms that are equivalent to those in humans, including late neurological degeneration, occurring in 3dr-4th decade in humans and at 8-10 months in the mouse model.

In earlier studies (ref 20,21) they showed that replacement therapy with human ceruloplasmin (Cp) in cpKO mice to some extend restored cerebral ceruloplasmin levels, and that was accompanied with amelioration of cerebral iron deposition, neurological symptoms, neuroinflammation, and neuro-degenerative phenotype. In the present study they examined the biodistribution of one i.v. injection of 64-Cu labelled human CP (64-Cu-Cp) in both cpKO and wildtype (WT) mice aged 6 or 10 months.

21 hours after the injection, PET-CT showed uptake of 64-Cu-Cp in several organs, including the brain, the heart and the liver. This was confirmed by analyzing the isolated organs after sacrificing the animals at the 21 hour time point.

They examined the biodistribution of 64-Cu-Cp and its variation according to genotype, age (6 months or 10 month) and sex.

The somewhat surprising finding was that distribution not only depended on genotype and age, as one might expect, but also sex.

In the WT mice the most striking differences were in the cerebellum. In the females, cerebellar uptake was clearly higher at 6 months than at 10 months, while the opposite was seen in males (lower at 6 months than at 10 months). As a result, at 6 months of age, the cerebellar uptake was higher in females than in males and vice versa at 10 months. Other sex dependent differences may have been overlooked due to low sample size, N=3 in each group.

In the cpKO mice with a somewhat larger sample size, the picture was slightly more detailed. Again, the most consistent signal was from the cerebellum and here with the complete opposite results: In female rats, cerebellar uptake was lower at 6 months than at 10 months while the opposite was true for males. Consequently, at 6 months of age, the cerebellar uptake was lower in females that males and vice versa at 10 months.

A less clear finding was from liver, with higher uptake at 6 months in males, and indications ([18F]-VC701uptake) of greater macrophage activation in males than females. However that was not confirmed histologically.

Taken together these findings question generalizations from WT to cpKO animals and from one sex to the other which may be important for future preclinical studies of Cp supplementation in ACP (- If the results can be generated from one species to another !).

The findings in the submitted paper predict that the therapeutic effect of CP replacement would be higher in cpKO male than cpKO female animals since the higher uptake in males occurs before neurological symptoms present and while the Cp distribution in females is most abundant after the time point where symptoms arise. In accordance, a re-analyzis of data from the two earlier studies showed a more consistent treatment effect in male rats than in females.

It is a very interesting study, well preformed and clearly presented. I have a few suggestions, the authors should consider.

Majors

1.The introduction can be shortened. It includes a comprehensive review of what is known about ACP and can be focused more on what is relevant as the background for the present study.

2.The methodology includes labeling of human CP by isotope transfer; human CP is exposed to a medium containing 64-Cu and apparently 64-Cu and cold Cu wap positions. That was well documented (appendix). In that case 64-Cu may also be replaced with cold Cu in plasma. By use of PET-CT they followed the distribution of 64-Cu. How can they know that the isotope has not left the CP molecule at that time point ?

4. The results may also be relevant for other diseases with low ceruloplasmin. In Wilson disease (inherited ATP7B deficiency), ceruloplasmin is also low, and damage to basal ganglia and neurological symptoms typically develop in the 2nd-3rd decades. There is accumulation of copper in the brain, but could the ceruloplasmin deficiency also be important ? In Wilson disease the lack of ceruloplasmin is caused by the ATP7B deficiency since ATP7B is involved in the incorporation of copper into CP. Would CP replacement theoretically help these patients ?

5. The choroid plexus is located close to cerebellum. How can the authors be sure that the signal from cerebellum was not from the choroid plexus ?

6.The sample sizes were quite small and should be discussed as a possible limitation.

7. The study examined biodistribution after a single injection. The biodistribution after longtime treatment may be different. Discuss that as a limitation. However in earlier studies they measured human Cp in the animal brains after longterm treatment; would it be possible to look for sex differences in those data ?

Minors.

Fig 1. b and c. Not quite clear what “male-female pool” is. I assume it is all animals (males+females).

Fig 1. Legend. Three differ -> three different

Page 20, line 3. “it results in that in the” . I suggest “appears” is better than “results”.

Page 22, bottom line. “numerousness “ could be substituted with “sample size”

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The authors have done an excellent job, thoroughly addressing all the points and strengthening the paper.

Reviewer #2

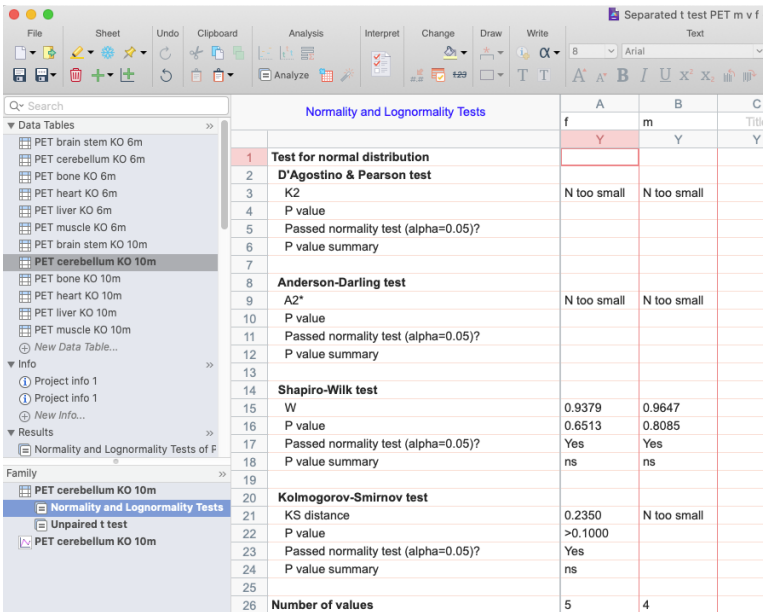
(Remarks to the Author)
I think the authors did a great job and responded well to the questions raised by me and the other reviewer. I have no further comments

Point by point response to the Reviewers' comments

Reviewer #1 (Remarks to the Author):

In this manuscript the authors follow up on their previous studies on the use of Cp for enzyme replacement therapy in the mouse model of aceruloplasminemia. The novelty of the present work compared to their previous CommBiol paper is the finding that there appear to be sex-related changes in the distribution of Cp administered to CpKO mice. Male and female Cp-KO mice of 6 and 10 months of age are analyzed and compared to wild type mice for distribution of 64Cu-labelled Cp evaluated by PET/CT and ex vivo. The results are very interesting and potentially relevant for design of sex-specific therapy for aceruloplasminemia patients.
Authors' comment: we thank the Reviewer for her/his positive judgement on the interest and potential relevance of our work, and for highlighting to us the weak points of the work.

The small number of animals makes some of the statistics a bit tricky (e.g., significant differences in 64Cu uptake in cerebellum at 10 vs 6 months of age appear to be due to a single individual, see fig 2b,d and fig 3b,c) but trends are nevertheless visible.
Authors' answer: we are aware that the small size of the compared groups could be a possible limitation of our study, but this was the results of several reasons. First of all, we have to accomplished to the Italian and European laws that require the use of the minimal number of animals, according to the 3Rs rules (Replacement, Reduction, Refinement). Thus, based on the expected theorical differences and the experience acquired with several other studies performed by our nuclear medicine specialists in animal PET, the sample size was calculated with the G-Power software. The calculation was done for the comparison of two mean of repeated measurement from two independent groups with a difference of at least twice the value of the SD, using the alpha error=0.05, the 80% of statistical power, two-tailed assay and effect size 2. Applying these parameters for the students'-t test the sample size resulted to be n=6 mice per group. Therefore, we obtained from the Italian Ministry of Health the approval for a protocol with 6 mice per group, no more. However, since the aceruloplasminemia is an accumulation disease, the mice need to be aged in order to develop the symptoms, and it occurs that during aging till 10 months some animals die for aceruloplasminemia-unrelated reasons (e.g. cannibalism, development of spontaneous tumors). These are the reasons why the cpKO groups are not homogeneous and limited in size.
Concerning the WT mice, they were used in the study to be sure that what we were seeing on the cpKO mice was genotype specific, so we kept their number to the minimum (see first authors' comment to Reviewer #2).
However, despite the small size of the samples we don't believe that the statistics we presented is "a bit tricky" and that the conclusions inferred might be "due to a single individual".
We verified that the individual with highest value (0.887) in fig. 2b and 2d is not an out-layer since the distribution of the values in the female group at 10 months is considered normal with both Shapiro-Wilk test and Kolmogorov-Smirnov test.



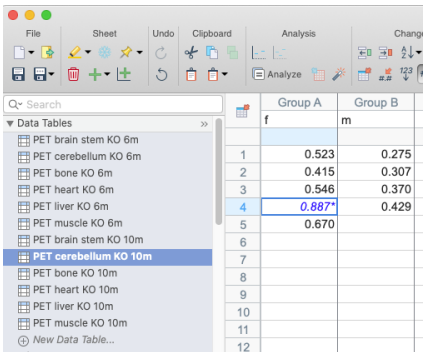
Normality and Lognormality Tests

		A	B
		f	m
		Y	Y
1	**Test for normal distribution**		
2	**D'Agostino & Pearson test**		
3	K2	N too small	N too small
4	P value		
5	Passed normality test (alpha=0.05)?		
6	P value summary		
7			
8	**Anderson-Darling test**		
9	A2*	N too small	N too small
10	P value		
11	Passed normality test (alpha=0.05)?		
12	P value summary		
13			
14	**Shapiro-Wilk test**		
15	W	0.9379	0.9647
16	P value	0.6513	0.8085
17	Passed normality test (alpha=0.05)?	Yes	Yes
18	P value summary	ns	ns
19			
20	**Kolmogorov-Smirnov test**		
21	KS distance	0.2350	N too small
22	P value	>0.1000	
23	Passed normality test (alpha=0.05)?	Yes	
24	P value summary	ns	
25			
26	**Number of values**	5	4

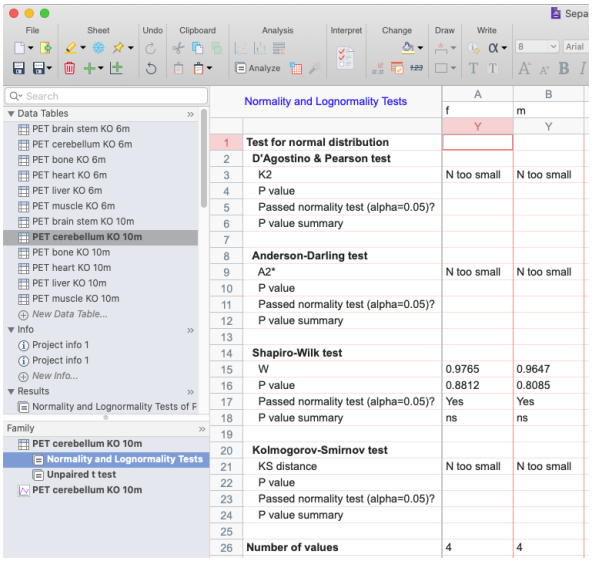
(screenshot of the analysis performed with Prism software)

Moreover, for the Reviewer evaluation only, we re-analyzed the cerebellum results that he/she indicated as possibly critical (fig 2b, d and fig. 3b, c) eliminating the highest values.

In the case of Fig. 2b in the absence of the highest 0.887 value, the distribution is still considered normal by Shapiro-Wilk test (eliminating one value the K-S test was not suitable) and the p value of the students' t-test is improved, therefore indicating that the statistical results were trustable and not due to a single individual.

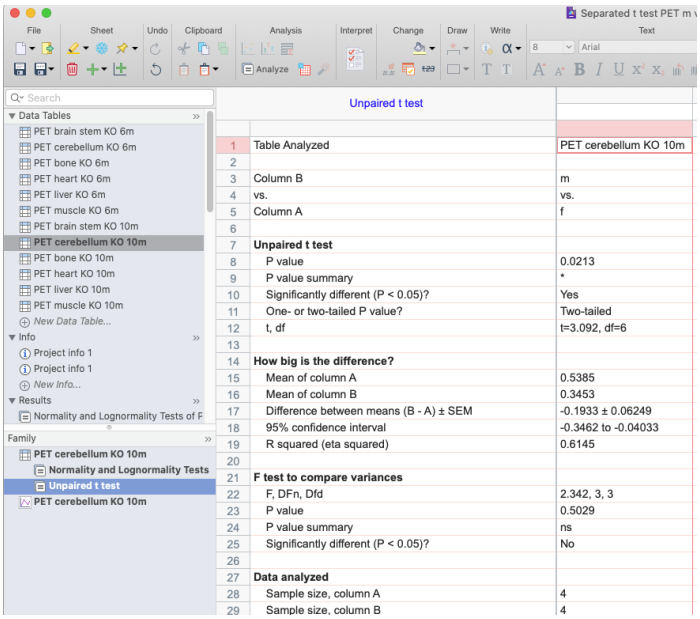


	Group A	Group B
	f	m
1	0.523	0.275
2	0.415	0.307
3	0.546	0.370
4	*0.887**	0.429
5	0.670	



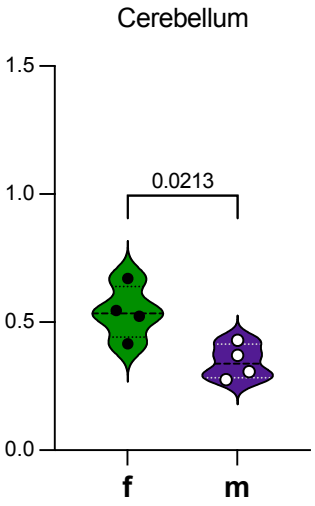
Normality and Lognormality Tests

		A	B
		f	m
		Y	Y
1	**Test for normal distribution**		
2	**D'Agostino & Pearson test**		
3	K2	N too small	N too small
4	P value		
5	Passed normality test (alpha=0.05)?		
6	P value summary		
7			
8	**Anderson-Darling test**		
9	A2*	N too small	N too small
10	P value		
11	Passed normality test (alpha=0.05)?		
12	P value summary		
13			
14	**Shapiro-Wilk test**		
15	W	0.9765	0.9647
16	P value	0.8812	0.8085
17	Passed normality test (alpha=0.05)?	Yes	Yes
18	P value summary	ns	ns
19			
20	**Kolmogorov-Smirnov test**		
21	KS distance	N too small	N too small
22	P value		
23	Passed normality test (alpha=0.05)?		
24	P value summary		
25			
26	**Number of values**	4	4

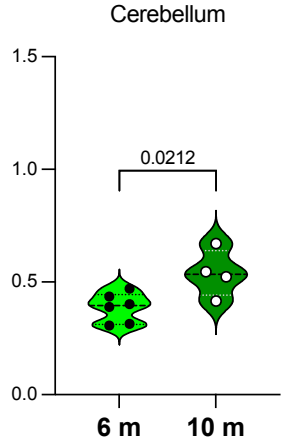


Unpaired t test

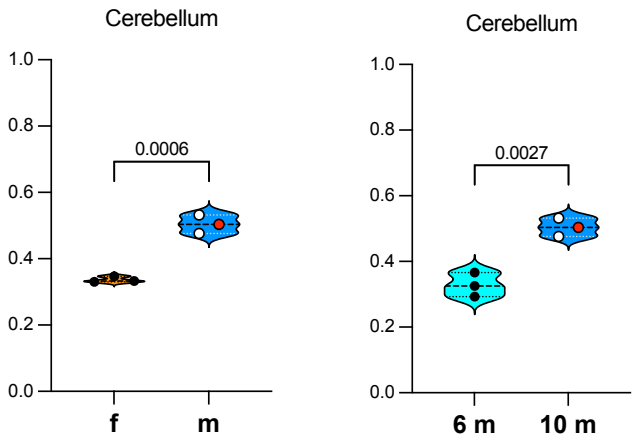
1	Table Analyzed	PET cerebellum KO 10m
2		
3	Column B	m
4	vs.	vs.
5	Column A	f
6		
7	**Unpaired t test**	
8	P value	0.0213
9	P value summary	*
10	Significantly different (P < 0.05)?	Yes
11	One- or two-tailed P value?	Two-tailed
12	t, df	t=3.092, df=6
13		
14	**How big is the difference?**	
15	Mean of column A	0.5385
16	Mean of column B	0.3453
17	Difference between means (B - A) ± SEM	-0.1933 ± 0.06249
18	95% confidence interval	-0.3462 to -0.04033
19	R squared (eta squared)	0.6145
20		
21	**F test to compare variances**	
22	F, DFn, Dfd	2.342, 3, 3
23	P value	0.5029
24	P value summary	ns
25	Significantly different (P < 0.05)?	No
26		
27	**Data analyzed**	
28	Sample size, column A	4
29	Sample size, column B	4



Along the same reasoning, the following is the result for fig 2d, in which the highest value was removed. Also in this case the statistical significance was preserved, indicating that the statistic values showed for the results presented in the manuscript are trustable even in the case of smaller number of mice.



In the case of the re-evaluation of Fig 3b and d, to keep the number of individuals to 3, the minimum necessary for the statistical analysis, the highest value of WT male mice at 10 months (0.726) was substituted with the average of the other two values (0.504, red dot in the following graphs). Also in these cases, the results indicated that the highest values were not forcing the statistical significance of the data presented.



Though, as requested by Reviewer #2 (point 6), the limitation of the study due to the small size of the groups is now reported in the discussion section of the revised version of the manuscript (page 18; "*Nevertheless, a possible limitation of our study is the low sample size that may have overlooked some other sex- or age-dependent differences*.").

The discussion could be expanded by adding information (if available) on the health of the CpKO mice: are there any sex-specific differences/signs of neurodegeneration at 10 months of age?

Authors' answer: As reported in the original discussion, so far in the literature the date regarding the pathological features of cpKO mice were not processed or interpreted considering the two sexes separately. Therefore, we re-analysed the data of cpKO mice from our previous work (*Zanardi et al Comm. Biol 2024*) comparing the pathological parameters of cpKO female mice to the parameters of male mice. We excluded from the analysis the features known to be sex-associated, like body and organs weight, and adipose tissue volume. The analysis was done for the animals at 6 and 10 months of age directly comparing the individual parameters.

For parameters associated to the neurodegeneration/neuroinflammation we did not find significant differences between males and females of cpKO mice, both at 6 and 10 months of age, except for the iron deposition in the choroid plexus which was higher in males than females in older mice. Similarly, for liver damage-associated parameters, the unique difference was in the F4/80 antigen staining, a feature referred to macrophage infiltration and Kuppfer cells activation, which resulted to be higher in males than females at 10 months of age. This observation confirmed the data reported in the original version of the manuscript, supporting the conclusion that larger uptake of ^{64}Cu-CP in females than males at 10 months of age was not correlated to a higher liver inflammation.

The major differences between the two groups of animals were found in the hematological parameters associated to the erythropoiesis. Indeed, several of these parameters were significantly different between cpKO males and females at 6 months of age, with a worst pathological condition associated to the erythropoiesis in females than males. The re-analysis indicated that cpKO female mice showed a consistent earlier or worst dysregulation in the erythropoiesis than males at the onset of the disease symptoms. This is an interesting observation because the anemia-related symptoms were reported as the initial clinical signs also for females in humans (*ref. 44 in the original manuscript, Ketata, Rare, 2023*). Thus, our re-analysis confirmed that the cpKO

mouse is a good model for the aceruloplasminemia disease also from the sex-related differences point of view. The difference between males and females cpKO mice in erythropoiesis-associated hematological parameters was maintained at 10 months only for few of them, suggesting a worsening of the erythropoiesis symptoms in males during the disease progression.
The results of the re-analysis of the pathological features in males versus females have been introduced in the Results section (pages 15-16) and commented in the Discussion section of the revised version of the manuscript (page 21). The values and statistics of the significant parameters are presented as new Supplementary Table 1.

Another point that deserves some discussion is related to the fact that the authors assume that 64Cu is a proxy for Cp. In the previous study (CommBiol 2024) Cp was measured by ELISA on ex vivo samples, is it possible to confirm the presence of the protein also in this case? In the literature there is evidence that Cp may be a source of copper for cells and that only 64Cu is taken up in cells incubated with double labelled Cp (64Cu and 125I) (some papers published around 1990 and more recently in 2016), so this should be discussed.

Authors' answer: We agree with the Reviewer that this might be a critical point for the conclusions of the work, and really appreciated the experimental suggestion that she/he gave us. We now measured by ELISA the presence of the CP protein in the brain lysates of the *cp*KO mice that were injected with the radiolabeled CP. The results clearly showed that after 21 h from the administration the CP protein is present in the brain of the mice both at 6 and 10 months of age. This indicates that the radioactive signals measured were a real proxy for the presence of the CP protein in the organs (see also the answer to the major point 2 from Reviewer #2 addressing the possible 64Cu swapping with cold Cu). This new result has been added in the revised version of the manuscript as Supplementary Figure 2, reported and commented in the Results and Discussion sections (page 9 and page 18, respectively). Materials and Methods section has been implemented with the ELISA procedure.
As highlighted in the literature suggested by the Reviewer, CP may be a source of copper for cells and the signal from 64Cu that we measured by PET and ex-vivo might be from the intracellular 64Cu internalized upon release from CP. Indeed in vivo imaging cannot discriminate between free 64Cu and CP-bound 64Cu, or between plasma membrane or cytosolic signals. Nevertheless, considering the intracellular Cu exchange mechanism described in the literature, the close proximity/binding of CP with the cell membrane is necessary for the release and internalization of 64Cu to occur. This in turn implies that the CP we injected in mice must have reached the target organs, therefore supporting the concept that our results reflect the biodistribution of the administered CP. This concept has been now introduced in the Discussion section of the revised version of the manuscript (page 18).

Minor points
1. please uniform radioactivity units: in the labeling reaction the authors indicate that 2 mCi of copper bound to Cp (0.2 mg), then it is stated that ca. 5 MBq of 64Cu-Cp are injected in the caudal vein of mice. Please indicate the amount of protein that is actually employed and how does it compare to the previous 2018 and 2024 studies.

Authors' answer: the radioactivity units have been uniformed along the entire text as to be MBq, accordingly to the international rules. The amount of ceruloplasmin injected with the 5 MBq equivalent and its relationship with the quantities administered in the previous works, has been reported in the Materials and Methods section (page 24; "*The day of the experiment, animals were injected in the caudal vein with 5.12±0.25 MBq of [^{64}Cu]-CP which corresponded to about 14 µg of CP and was on average 12.5 folds lower than the amount intraperitoneally administered to the mice in our previous works*.").

2. in figure S1b the 'brain' panel is duplicated and the 'eyes' panel is missing
Authors' answer: the panel has been replaced with the correct one.

Reviewer #2 (Remarks to the Author):

The authors study a mouse model (cpKO) of aceruloplasminemia (ACP). These mice develop symptoms that are equivalent to those in humans, including late neurological degeneration, occurring in 3dr-4th decade in humans and at 8-10 months in the mouse model.

In earlier studies (ref 20,21) they showed that replacement therapy with human ceruloplasmin (Cp) in cpKO mice to some extend restored cerebral ceruloplasmin levels, and that was accompanied with amelioration of cerebral iron deposition, neurological symptoms, neuroinflammation, and neuro-degenerative phenotype. In the present study they examined the biodistribution of one i.v. injection of 64-Cu labelled human CP (64-Cu-Cp) in both cpKO and wildtype (WT) mice aged 6 or 10 months.

21 hours after the injection, PET-CT showed uptake of 64-Cu-Cp in several organs, including the brain, the heart and the liver. This was confirmed by analyzing the isolated organs after sacrificing the animals at the 21 hour time point.

They examined the biodistribution of 64-Cu-Cp and its variation according to genotype, age (6 months or 10 month) and sex. The somewhat surprising finding was that distribution not only depended on genotype and age, as one might expect, but also sex.

In the WT mice the most striking differences were in the cerebellum. In the females, cerebellar uptake was clearly higher at 6 months than at 10 months, while the opposite was seen in males (lower at 6 months than at 10 months). As a result, at 6 months of age, the cerebellar uptake was higher in females than in males and vice versa at 10 months. Other sex dependent differences may have been overlooked due to low sample size, N=3 in each group.
Authors' comment: the aim of the study was to define the biodistribution of administered CP in the *cp*KO model of the disease because these animals are the target of the therapeutic approach. Indeed, WT mice, as well as healthy human subjects, will never be treated with ceruloplasmin, so the results in WT mice are not relevant from the therapeutic point of view and nor were for the main message of the

manuscript, namely the sex-dependent differences in CP-biodistribution in cpKO mice. The WT mice were used in the study to be sure that what we were seeing on the cpKO mice was genotype specific, therefore their number was kept to the minimum (see also the first answer to Reviewer #1).
We agree with the Reviewer that with the limited number of individuals some differences may have been missed, and as suggested in point 6, a sentence underlining this limitation of the study has been introduced in the discussion (page 18; "*Nevertheless, a possible limitation of our study is the low sample size that may have overlooked some other sex- or age-dependent differences*.").

In the cpKO mice with a somewhat larger sample size, the picture was slightly more detailed. Again, the most consistent signal was from the cerebellum and here with the complete opposite results: In female rats, cerebellar uptake was lower at 6 months than at 10 months while the opposite was true for males. Consequently, at 6 months of age, the cerebellar uptake was lower in females that males and vice versa at 10 months.

A less clear finding was from liver, with higher uptake at 6 months in males, and indications ([18F]-VC701uptake) of greater macrophage activation in males than females. However that was not confirmed histologically.
Authors' comment: In our study we performed PET imaging with [18F]-VC701 only in 10 months-old animals (Figure 4).
The analysis of [18F]-VC701 tracer uptake was done with the aim to define whether the different uptake of ^{64}Cu-CP observed in the liver of males and females *cp*KO mice at 10 months of age was merely depending on the inflammatory status of the organ (e.g. by increased vascular permeability or general metabolic demanding). The results obtained indicated that this was not the case because at 10 months of age, despite the larger ^{64}Cu-CP uptake in liver (Figure 2b), the females showed significant lower values than males of the two parameters considered as proxy of the inflammation, namely [18F]-VC701 uptake (Supplementary Figure 3a) and F4/80 macrophage and Kupffer cells marker staining (Figure 5c). The immune-histological analysis performed in liver at 6 months of age confirmed this conclusion since, at comparable level of F4/80 staining, cpKO males displayed significantly larger uptake of ^{64}Cu-CP than females (Figure 5b and Figure 2a, respectively).
The same conclusions, namely that ^{64}Cu-CP uptake is not depending on the organ's inflammatory status, can be inferred by comparison of the [18F]-VC701 uptake in the cerebellum of *cp*KO mice at 10 months of age that was similar in males and females (Figure 4a) despite the larger uptake of ^{64}Cu-CP observed in females (Figure 2b).
To make the aim of this part of the study, its results and the conclusions clearer, we changed the subheading of the paragraph in the Results section (page 10 old subheading "*At 10 months of age cpKO male mice show higher liver inflammation than females*", new one "*Larger ^{64}Cu-CP uptake in the liver of cpKO females than males at 10 months of age is not depending on liver inflammation*."), and we added new sentences both in the Results and Discussion sections (page 13-14; page 20, respectively) of the revised version of the manuscript.

Taken together these findings question generalizations from WT to cpKO animals and from one sex to the other which may be important for future preclinical studies of Cp supplementation in ACP (- If the results can be generated from one species to another

!).
Authors' comment: we agree with the cautiousness expressed by the Reviewer (in brackets). As rightly pointed by the Reviewer, sex differences in CP bioavailability observed in mice could not be directly translatable to patients. This point was already faced in the "Discussion" section of the original version of the manuscript (page 19 in the revised version of the manuscript).

The findings in the submitted paper predict that the therapeutic effect of CP replacement would be higher in cpKO male than cpKO female animals since the higher uptake in males occurs before neurological symptoms present and while the Cp distribution in females is most abundant after the time point where symptoms arise. In accordance, a re-analyzis of data from the two earlier studies showed a more consistent treatment effect in male rats than in females.

It is a very interesting study, well preformed and clearly presented. I have a few suggestions, the authors should consider.
Authors' comment: we thank the Reviewer for the positive evaluation of our work and for the suggestions.

Majors

1.The introduction can be shortened. It includes a comprehensive review of what is known about ACP and can be focused more on what is relevant as the background for the present study.
Authors' answer: The Introduction section has been shortened as suggested

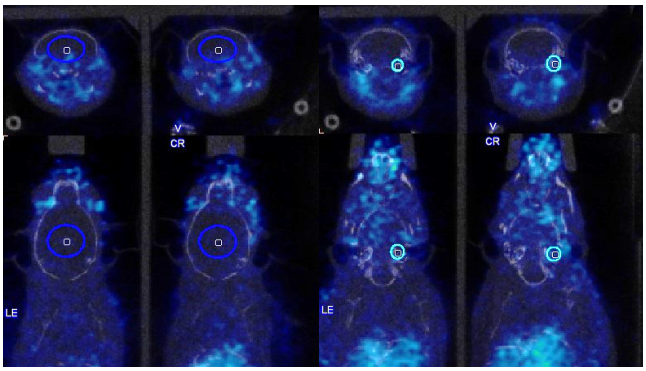
2.The methodology includes labeling of human CP by isotope transfer; human CP is exposed to a medium containing 64-Cu and apparently 64-Cu and cold Cu wap positions. That was well documented (appendix). In that case 64-Cu may also be replaced with cold Cu in plasma. By use of PET-CT they followed the distribution of 64-Cu. How can they know that the isotope has not left the CP molecule at that time point ?
Authors' answer: This is a very important point, indeed we can't exclude a priori that part of the signal that we measured by PET-CT and ex-vivo is from the 64Cu spontaneously released or swapped with cold Cu from labelled CP molecules. However, the original old and more recent literature reported that while the reversible dissociation of Cu from CP occurs *in vitro*, and is exploited for CP labelling, the exchange of the Cu incorporated in the CP with ionic Cu does not occur *in vivo*, where the Cu enters in the CP only during the synthesis of the protein and remains associated to it throughout its life span (*Sternlieb et al. J Clin Invest 1961; Percival & Harris, Am J Physiol 1990; Ramos et al. PLOSone 216*). The major differences between in vivo and in vitro conditions are: 1) that Cu swapping occurs in vitro because the environment is reducing (pH 5.6 and presence of ascorbic acid), which is not the case in vivo in the blood; 2) free Cu as ionic Cu is extremely low in serum, practically nonexistent. The vast majority of Cu in blood is bound to transporter proteins (also in cpKO mice), thus the swap should occur between Cu atoms coordinated by proteins which should implies a slower kinetics of exchange.

For these reasons we are convinced that the spontaneous swap in vivo between cold Cu and 64Cu ions within CP molecules was highly unlike. The release of radiolabeled Cu from CP has been demonstrated to occur in vitro in cell culture via a regulated process on the cell membrane, which requires the close proximity/binding of CP with the membrane, the presence of a reductase (necessary for Cu ions reduction, which is mandatory for their release from CP, as occurs for the in vitro labelling) and the presence of Cu-transporter on the cell membrane necessary for internalization of Cu (*Percival & Harris, Am J Physiol 1990; Ramos et al. PLOSone 216*). If Cp may be a source of copper for cells through this CP-mediated mechanism also in vivo has not been demonstrated so far. Nevertheless, it would imply that, even if the radioactive signal we measured in the different organs is from 64Cu released from CP and incorporated in the cells, the injected CP must have reached the target organs, thus our results reflect the biodistribution of the administered CP.
Furthermore, as suggested by Reviewer #1, we now measured by ELISA the presence of the CP protein in the brain of the *cp*KO mice that were injected with the radiolabeled CP. The results clearly indicated that the CP protein is present in the brain of the mice both at 6 and 10 months of age after the administration. This indicates that the radioactive signals that we were measuring is a real proxy of the CP protein, and that the contribution of isotope swapping, if occurred, was marginal (see also answer to third major question from Reviewer #1). This result has been added in the revised version of the manuscript as Supplementary Figure 2, reported and commented in the Results and Discussion sections (page 9 and page 18, respectively).

4. The results may also be relevant for other diseases with low ceruloplasmin. In Wilson disease (inherited ATP7B deficiency), ceruloplasmin is also low, and damage to basal ganglia and neurological symptoms typically develop in the 2nd-3rd decades. There is accumulation of copper in the brain, but could the ceruloplasmin deficiency also be important ? In Wilson disease the lack of ceruloplasmin is caused by the ATP7B deficiency since ATP7B is involved in the incorporation of copper into CP. Would CP replacement theoretically help these patients ?
Authors' answer: this is a good observation to which we are aware. Indeed, to address these questions we have a funded ongoing project on this topic in which we are treating with CP the ATP7B-/- mice model of Wilson. The concept that CP-replacement therapy could also be beneficial in other diseases in which the ceruloplasmin level is decreased (e.g. Parkinson, Alzheimer, Wilson, etc..) has been already claimed in the discussion of our recent work published in Communications Biology (*Zanardi et al 2024*), thus to avoid redundancy was not re-proposed in this manuscript.

5. The choroid plexus is located close to cerebellum. How can the authors be sure that the signal from cerebellum was not from the choroid plexus ?
Authors' answer: We analyzed PET images by using the brain volume of the Allen Brain Atlas as reference space (https://atlas.brain-map.org/). According to this, we placed a Volume of Interest (VOI) in correspondence to a broad subcortical region for cerebrum signal evaluation (in blue on the left) and in a lateral region for cerebellum (in azure on the right), as represented in the pictures below. The localization of the VOI minimizes the possibility of selecting the choroid plexus.
However, due to the close proximity of the cerebellum and choroid plexus of the fourth ventricles, we can't completely rule out that part of the signal, detected in that region

was due to CP accumulation in the choroid plexus. A sentence underlining this uncertainty has been introduced in the Discussion (page 17).



6.The sample sizes were quite small and should be discussed as a possible limitation.
Authors' answer: The issue was also raised by the Reviewer#1. Please, see first answer to Reviewer #1.

7. The study examined biodistribution after a single injection. The biodistribution after longtime treatment may be different. Discuss that as a limitation. However in earlier studies they measured human Cp in the animal brains after longterm treatment; would it be possible to look for sex differences in those data ?
Authors' answer: we agree with the Reviewer on this comment, even because in our studies the single injection and long-term treatment differ on the way of administration. The single injection was done intra-venous while the long-term treatment was done by intraperitoneal administration, since intravenous was not practicable for repeated injections. Moreover, we are grateful to Reviewer for her/his suggestion. Indeed, we re-analysed the data of CP detected by ELISA in the brain of cpKO mice long-term-treated with CP in our previous work considering males *versus* females. The results showed that CP accumulation in the brain at the end of the long-term treatment was greater in the female mice, which is analogous to the larger accumulation observed in the brain of females after a single injection at 10 months of age, thus suggesting similar biodistribution both in long-term administration and single injection.
The possible differences in biodistribution between the single injection and long-term treatment as limitation of the study have been now commented in the Discussion section of the revised manuscript, also in the light of the new results of the re-analysis performed on the data from the previous long-term treatment study (see page 20-21).
The results of the re-analysis presented in the discussion have-been introduced as new Supplementary Figure 4.

Minors.

Fig 1. b and c. Not quite clear what "male-female pool" is. I assume it is all animals (males+females).
Authors' answer: the definition "male-female pool" has been substituted with "males+females" in the figure and in the figure legend.

Fig 1. Legend. Three differ -> three different
Authors' answer: We corrected the typo errors, as suggested.

Page 20, line 3. "it results in that in the" . I suggest "appears" is better than "results".
Authors' answer: We corrected the typo errors, as suggested.

Page 22, bottom line. "numerousness " could be substituted with "sample size"
Authors' answer: We substituted with " to increase sample size", as suggested.