Author's response to reviews

Title: Suckling A Protein-Restricted Rat Dam Leads To Diminished Albuminuria In Her Male Offspring In Adult Life: A Longitudinal Study

Authors:

Clive J Petry (cip1002@cam.ac.uk)
Bridget J Jennings (bridget.jennings@mhra.gsi.gov.uk)
Lynwen A James (Lynwen.James@ucb-group.com)
Nicholas C Hales (cnh1000@cam.ac.uk)
Susan E Ozanne (seo10@cam.ac.uk)

Version: 3 Date: 1 August 2006

Author's response to reviews: see over
Dear Dr. Phillips,

Re: MS: 1264462125998106 Clive J Petry et al.
Suckling A Protein-Restricted Rat Dam Leads To Diminished Albuminuria In Her Male Offspring In Adult Life: A Longitudinal Study

Thank you for giving us the opportunity to respond to the reviewer’s comments and concerns about the above manuscript (the revised manuscript is submitted along with this covering letter). We have now thoroughly revised the manuscript according to these comments (changes to the manuscript are outlined below and are shown in a blue font), in particular due to the major concerns:

(i) we have changed the statistical analysis of the longitudinal part of the study to use repeated measures ANOVA. This has allowed us to present estimated marginal geometric means and to compare the individual groups to each other by post-hoc testing.

(ii) we have removed the histological analysis completely from the manuscript as it was performed at the end of the study (without any control kidneys available), it was
underpowered to really see anything (despite the significant difference in its preliminary findings), it was subjective and was only performed at a very superficial level.

We have also checked that the manuscript is now formatted correctly for your journal. We hope that you will now consider that the manuscript is suitable for publication as a Research Article.

Yours sincerely,

Clive Petry

Clive Petry on behalf of all of the co-authors
Reviewer's report
Title: Suckling A Protein-Restricted Rat Dam Leads To Diminished Albuminuria In Her Male Offspring In Adult Life: A Longitudinal Study
Version: 1 Date: 12 May 2006
Reviewer: Mary Black

Reviewer's report:
General
This study looked at the effect of maternal protein restriction in utero or during lactation on albuminuria and creatinine levels later in life. Although there is mention that renal pathology was assessed, the details of how this was assessed and the reporting of the results were inadequate.

Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)

Methods:
Many important details have been omitted from the Methods section. This section needs to be expanded.

The methods section has now been expanded in response to these concerns.

Animals: The protocol used in the animal studies is not clear. For instance, why were there 24 control offspring, 16 recuperated offspring and 20 PLP offspring? If there were 5 dams fed the low protein diet during pregnancy and 4 males were used per litter doesn't this mean that there should have been 20 recuperated offspring?

The number of litters and animals from each litter used has now been clarified (page 5). The reviewer is correct in her assumption that there were some apparently missing animals from the PLP group. The reasons for this have now been explained in the text (page 5, lines 5-7), namely that one litter randomly had only 2 male pups (which is not that uncommon in Wistar rats) and one mother displayed some infanticide behaviour (which again is not that uncommon, see reference 8).

Also, were the control offspring cross-fostered? Indeed, it is important that the controls were treated in the same way as the other groups.

The experiment was designed to compare rat offspring in adult life who had been exposed to different maternal diets in utero and whilst suckling, it was not designed primarily to test the effects of being cross-fostered by mothers consuming different diets (in fact the reason for cross-fostering rather than just swapping the diets of the birth mother was so that there was not a lag phase where the effects of the new diet are only slowly being felt). Also relative to the longevity of the control rats, PLP rats tend to have a longer lifespan and recuperated rats a shorter one (Jennings et al., FEBS Lett. 1999; 448:4-8) which does not suggest a major effect of exposure to the maternal control diet in early life on longevity. Our control offspring were therefore not cross-fostered. We recognise that cross-fostering the control group would have been a valid experimental design, although for the reasons outlined above we did not follow it. Additionally it may have been difficult to get this procedure licensed as the Home Office would probably have considered it unnecessary.
In my opinion, the decision to cease experiments at the time (10 months) when the first rat died was unusual and not well-justified. What did that rat die from? Ten months of age is far from the normal life span of a rat. I would expect that rats would normally live at least twice as long as this.

The way that the longitudinal urine albumin data was analysed requires a “full” data set, if data is missing all the data from that particular animal is excluded. The experiment therefore effectively finished once the first animal had died as the data set would have otherwise become more and more fragmented as more animals died.
See reference 2 for a survival curve from a previous study using this same experimental protocol (diets, strain of rat and type of animal facility used to house the rats) in a longevity experiment, where at 10 months of age only 75% of the recuperated animals were still alive (it is therefore not surprising that in our study of 16 original recuperated animals it was one of these animals that died first). Admittedly survival is relatively poor in these animals but that may also relate to the richness of the weaning diet used (which contributes to their relatively excessive weight gains).

Laboratory Analyses: Creatinine was measured in the plasma and in the urine. What does the albumin/creatinine ratio represent? How was the creatinine clearance determined? These details should be included in the methods or references detailing these techniques should be referred to.

Creatinine is a metabolite of creatine from the muscle and broadly speaking is secreted in relatively constant amounts throughout the day. It is very commonly used in clinical biochemistry as a marker of how concentrated urine is. Presenting the albumin/creatinine ratio therefore does not depend upon gaining an accurately timed urine sample or one that has been accurately sampled and has an accurate volume measurement (which are frequent causes of error in results from 24 h. urine specimens). Because of these factors the albumin/creatinine ratio is frequently used to quantify albumin excretion in early morning samples from people with diabetes to screen for microalbuminuria (see e.g. Marshall SM, Screening for microalbuminuria: which measurement? Diabet Med. 1991; 8:706-11). Whilst most people consider it the best way of presenting albumin excretion data, its drawback is that extra laboratory error can be introduced into the result because of the extra measurement (i.e. creatinine) and calculation that are required. We have therefore decided to present our albumin excretion data both as a timed excretion rate and as a ratio against creatinine, so that it is evident that the significant results that we found were not unduly influenced by the shortcomings in either method of presenting albumin excretion. In response to the concerns raised by reviewer 1 we have now included the method of calculating creatinine clearance (which we used as an estimate of glomerular filtration rate; page 6, lines 9-12) and stated the advantages and shortcomings of both ways of presenting albumin excretion data (page 6, lines 18-20).

If there were 16 or more animals in all experimental groups, why weren't all the kidneys analysed at the termination of the experiment? In particular, why were the number of animals chosen to study kidney pathology different between the groups and why were only 5 kidneys from the recuperated animals investigated. For morphological/pathological analyses I would expect that you would need 8 to 10 kidneys per group. Was a power analysis performed to determine how many kidneys were to be assessed? Please elaborate on what parameters were investigated and the standard deviations associated with these techniques.
There is insufficient information to determine whether the pathological analyses of the kidneys were conducted correctly. When referring to pathological lesions in the kidneys what specifically was looked at and what was the criteria for classification of the lesions as
minimal to mild, moderate or severe? Also, how were the kidneys sampled? How many sections and fields of view were assessed? It is essential that this information is provided. It is deduced from the results section that many characteristics of renal pathology were investigated such as focal segmental sclerosis, tubular dilation, tubular atrophy, mononuclear cell inflammation, interstitial fibrosis, cell hyperplasia in the distal tubules and interstitial inflammation. In my opinion to adequately address many of these pathologies, specific histological stains or immunohistochemistry should have been utilised and simple analysis of haematoxylin and eosin stained sections would be inadequate.

As stated in the opening comments of this covering letter to the editor, the histological analysis in this study was performed at a very superficial level at the end of the study (at which point there were no kidneys available from controls). It was originally included in the manuscript due to the supporting evidence it gave, albeit in a very preliminary fashion, to the suggestion that the recuperated animals displayed more renal pathology than the PLP animals. However, due to the concerns raised by reviewers 1 and 2, including those above, we have now removed all reference to the histological analysis from the manuscript.

Results:
For all data written into the text the means ± SEM should be included.

Much of the data in this study were not normally distributed and required either log-transformation prior to statistical analysis or to be analysed using a non-parametric test. We therefore feel that it is inappropriate to present the data as means ± SEM, as these would be unduly influenced by the data’s skewed distribution. For log-transformed data it is more appropriate to back transform the mean of the log-transformed data after the statistical analyses have been performed, hence we have presented the geometric mean throughout along with a confidence interval as a guide to the level of assurance in that geometric mean. Reviewer 3 (who was particularly interested in reviewing the statistical aspects of the study) did not comment on the way that the results were presented so presumably was happy with it.

The way the results are written it is difficult to differentiate between which groups the significant difference lies. For instance, were the daily albumin excretions significantly different between the controls and the recuperated groups? Likewise were the daily albumin excretions different between the PLP and control groups? It would be better if the albumin data and the plasma creatinines, urine creatinines and creatinine clearance data was presented in table form with symbols showing between which groups the significant differences were detected.

The trouble with putting too much data into tables is that it breaks the flow of being able to read the text, as the reader keeps having to refer to the table and then go back to the text. To overcome this concern raised by reviewer 1, however, we have made further use of post-hoc testing to compare individual groups (as in page 8, lines 19-21 and 25-27 and page 9, lines 6-8) (this was not originally performed due to concerns about multiple testing).

I find it very difficult to understand why after 10 months of age the albumin excretion rates in the controls and recuperated animals was considerably reduced. Indeed, the results in the control animals do not fit in with previously published data from this group (with some of the same authors) published in the Am J Physiol Renal Physiol 2006. In that published study in control male Wistar rats the urinary albumin excretion per 24 hr was about 20 mg at 7 months of age and this had increased markedly to 80 mg at 15 months of age. To the contrary in this study, the urine albumin excretion per 24 hrs was about 80mg at 7 months of age and had dropped to less than 20 mg at 10 months of age. These findings in relation to their previous findings should have been discussed in the discussion. Also, they should have been further discussed in relation to other published studies.
The reviewer makes a valid point in highlighting the difference in urine albumin excretion rates between the current study and the paper recently published in the Am J Physiol Renal Physiol 2006. In that paper the animals were not exposed to the maternal diets used in the current study. The biggest difference between the two studies, however, is the environmental exposure that the animals had: in the study published in Am J Physiol Renal Physiol the animals were housed in individually ventilated cages in a ‘clean’ animal facility, whereas in the current study they were housed in a conventional non-barriered facility. Whilst the difference may seem trivial it has already led to substantial differences in lifespans of the rats (unpublished results). The animals housed in the ‘cleaner’ facility now have a much longer lifespan and so may only experience the effects associated with age when they are much older than when they are observed in animals housed in the conventional facility. It is entirely feasible that if the animals in the ‘cleaner’ facility had had their urine albumin excretion rates measured at an even older age (e.g. 22 months) they would have dropped in a similar manner to that observed in the current study. To clarify this in the manuscript, in the Animals section of the Methods (page 4, lines 11-12) we have now clearly stated that the animals were housed in a conventional, non-barriered facility and in the Discussion section (page 10, lines 18-24) we have explained the differences between the two studies.

The renal histology section should be re-written. If all the parameters (focal segmental sclerosis, dilated tubules, tubular atrophy, interstitial fibrosis etc.) were all assessed in this study they should have been recorded and assessed separately. For instance, were the kidneys from the PLP group more susceptible to a particular renal pathology? This data could be reported in table form. Also, light micrographs of representative pathologies would be useful.

As stated in the opening comments of this covering letter to the editor and above, the histological analysis in this study was performed at a very superficial level at the end of the study (at which point there were no kidneys available from controls). It was originally included in the manuscript due to the supporting evidence it gave, albeit in a very preliminary fashion, to the suggestion that the recuperated animals displayed more renal pathology than the PLP animals. However, due to the concerns raised by reviewers 1 and 2, including those above, we have now removed all reference to the histological analysis from the manuscript.

Abstract:
It is incorrect to conclude that the slower rate of shortening in kidney telomere lengths (and increased lifespan) in rats exposed to maternal protein restriction during lactation was associated with diminished albumin excretion rats when you did not look at these associations in this study. You can only make conclusions on the parameters and data you investigated. You also cannot make the final statement in the conclusion. Firstly, you did not look at whether maternal protein restriction leads to nephron damage in utero. Indeed, I don't think that it is generally considered that maternal protein restriction in utero leads to nephron damage in the fetus. It generally leads to reduced nephron endowment in the offspring but that does not imply that the nephrons are damaged.

We have now rewritten the Conclusions section of the Summary (page 3) to remove the implication about kidney telomere lengths. We agree that maternal protein restriction leads to reduced nephron endowment in the offspring at birth. The nephron damage found in rats
exposed to pre-natal maternal protein restriction (in later life) may well be just due to age-
associated wear and tear in animals that are higher risk because of their lower endowment,
however.

**Background:**
In the final paragraph of the Background, the aims were to investigate whether there were
differences in renal function and pathology in male rats that were exposed to maternal
protein restriction either pre-natally or whilst suckling. I do not think that renal function or
pathology were adequately assessed. In rat studies it is possible to measure a number of
parameters to assess renal function, such as glomerular filtration rate, renal blood flow,
renal vascular resistance, filtration fraction and urine flow rate. Since these measures were
not performed, it would be better to state the aims more specifically to indicate what
parameters were investigated. Also, and more importantly, renal pathology did not appear
to be adequately assessed.
However, the description of how renal pathology was assessed in the methods and the
outcomes were so poorly described that it is difficult to ascertain whether the analyses
were performed correctly and what was measured.

Once again as stated in the opening comments of this covering letter and above, the histological
analysis in this study was performed at a very superficial level at the end of the study (at which
point there were no kidneys available from controls). It was originally included in the
manuscript due to the supporting evidence it gave, albeit in a very preliminary fashion, to the
suggestion that the recuperated animals displayed more renal pathology than the PLP animals.
However, due to the concerns raised by reviewers 1 and 2, including those above, we have now
removed all reference to the histological analysis from the manuscript. We have also now
altered the stated aim of the study, to better reflect what we were actually trying to achieve
(page 4, end of first paragraph).

**Methods:**
Laboratory Analyses: I think it is appropriate to reference the techniques for the
immunosorbent assay for the measurement of albumin and to the Jaffe reaction for
measuring creatinine.

These have now been added to the manuscript (references 9 and 10).

**Discussion:**
First sentence: Second word should be deleted.

This has been carried out (page 9).

Most of the first paragraph describing results from a previous study should be either
reduced or deleted.

Most of the first paragraph has now been shortened (page 9) and merged with what was
originally the second paragraph.

Second paragraph: You cannot say that exposure to maternal protein restriction during the
first few weeks of life led to significant reductions in urinary albumin excretion at 3 months
of age. The changes in albumin excretion may be secondary to some other change in the
kidney that has subsequently led to the changes in albumin excretion. You can state that it
is linked but not that it led to the effects.

The only difference between the different groups of animals tested was the maternal diet(s) that
they were exposed to, i.e. they were from an inbred strain and so should have been almost
identical genetically and also their environmental exposures (other than the maternal diet
exposure and possibly the cross-fostering employed to alter that exposure) were exactly the same. Therefore we feel entirely justified in suggesting that it was the differences in maternal diets that the animals were exposed to that led to the differences in the urine albumin excretion rates, presumably through some kind of ‘programming’ mechanism. We have no doubt that there are intermediate steps in this process, however, and as an example of what may be happening we state (page 11, end of paragraph 2) that using this model PLP animals eat less than the other animals – a factor known to affect renal function. Just because there are programming mechanisms involved, however, does not alter the fact that it is the exposure to the maternal diet that ultimately causes the changes in urine albumin excretion.

It is imperative that the authors discuss their albumin excretion results (especially in the control animals) in context with other published findings, including studies from their own laboratory. Are there other studies that have shown that in control animals or normal subjects that albumin excretion is reduced with age?

See the response to the similar concern also from reviewer 1 raised earlier. It is also important to note that there are no other published studies that have taken a longitudinal approach to investigating rat urine albumin excretion rates in the same way that this study has.

Conclusions: Much of what is stated in the conclusions is speculative and cannot be directly determined by the results presented in this study.

We have now completely rewritten the Conclusions section (page 12) in response to this concern.

Discretionary Revisions (which the author can choose to ignore)

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions
Level of interest: An article whose findings are important to those with closely related research interests
Quality of written English: Acceptable
Statistical review: Yes
Declaration of competing interests: I declare that I have no competing interests.
**Reviewer's report**

**Title:** Suckling A Protein-Restricted Rat Dam Leads To Diminished Albuminuria In Her Male Offspring In Adult Life: A Longitudinal Study

**Version:** 1  **Date:** 5 July 2006  
**Reviewer:** Elena Zambrano  
**Reviewer's report:**

**General**

This is a very exciting and well written article that describes the effect of maternal low protein diet in the male rat offspring renal function. It explains possible mechanisms around the kidneys long-lasting protection against future age related diseases. The exposure to maternal protein restriction during lactation would thus prolong life due to the preservation of renal function. The results are interesting and make important contributions to science.

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**Major Compulsory Revisions (that the author must respond to before a decision on publication can be reached)**

I have found one major concern and think that it is definitely not publishable unless they clearly state short litter number. Please provide the n number of litters rather than pups.

The litter and pup numbers for each group of rats has now been presented (page 5), including an explanation as to why there were fewer recuperated animals than those in the other groups (page 5, lines 5-7).

In addition I would like the authors to give better information on which methods they were supported to perform the renal histology. It is necessary to define pathological lesions in kidney. They explained severe lesions in the results section, never defining between minimal to moderate lesions, I would rather them to describe it in the methods of pathological renal histology.

I found table 1 very hard to understand: where is the Control group? I assume there were no kidney lesions in that particular group, but I would like to have the whole comparison among groups in the table.

In addition, in table 1 the percentage of lesions does not apport anything, I prefer to have the % of animals in the whole group that have minimal or severe kidney lesions, and the % of animals with no lesions at all.

This table is qualitative observation (subjective) rather than quantitative.

As stated in the opening comments of this covering letter to the editor, the histological analysis in this study was performed at a very superficial level at the end of the study (at which point there were no kidneys available from controls). It was originally included in the manuscript due to the supporting evidence it gave, albeit in a very preliminary fashion, to the suggestion that the recuperated animals displayed more renal pathology than the PLP animals. However, due to the concerns raised by reviewers 1 and 2, including those above, we have now removed all reference to the histological analysis from the manuscript.

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**Minor Essential Revisions (such as missing labels on figures, or the wrong use of a term, which the author can be trusted to correct)**

Figure 1 shows the body weight in different groups, the symbols are indistinguishable between control and recuperated groups. I strongly suggest to make some changes that help to see both groups.
This is actually surprisingly hard to do since the body weights of the control and recuperated animals were indistinguishable from 3 weeks of age (therefore however you plot a graph one line would always be superimposed upon another). However by making the black squares (which represent the weights of the control animals) bigger we have tried to clarify this problem.

In the discussion they explained “The higher creatinine clearances at ten months of age in the recuperated (who may have reduced glomerular loads [ref 17-19]” parenthesis never end. In addition they can not assume with certainty their findings with the references cited, they could only suggest some similitudes.

An extra closing bracket has now been added (page 11, line 10). In addition the surrounding sentence has been modified to make it clearer that we are suggesting a possibility rather than making a statement.

Discretionary Revisions (which the author can choose to ignore)
Sometimes the authors refer control group, then post natal group and at the end the recuperated group (like in figure legend 2), but in the graph the order is different. Same observation in table 1. they ought to have same order at all times.

We have now modified the figure legend to comply with this suggestion (page 18).

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions
Level of interest: An article of importance in its field
Quality of written English: Acceptable
Statistical review: No
Declaration of competing interests: I declare that I have no competing interests
Elena Zambrano
Reviewer's report

Title: Suckling A Protein-Restricted Rat Dam Leads To Diminished Albuminuria In Her Male Offspring In Adult Life: A Longitudinal Study

Version: 1 Date: 17 July 2006  
Reviewer: Andrew Roddam

Reviewer's report:
This review considers only the statistical aspects of the manuscript for which I have only two comments:

1. At the top of page 8 it is shown that there is no difference between two groups without a p-value, either give the actual p-value or say p>0.05/p non-significant.

This has now been amended (page 7, line 18) by reporting the actual p-value.

2. I found the description of the linear regression analysis both in the methods section and in the results not very clear. Do you simply put all the observations (log-transformed) into a regression model and then adjust for age and animal. Is the adjustment for animal a regression adjustment or does it account for the natural correlation that will exist in measurements taken from the same animal, i.e. does it correct the standard errors for repeated observations? Is the age adjustment a linear term - the relationship shown in Figure 2 (a & b) does not look linear - have non linear terms being used? Alternatively was age entered in as a series of discrete variables? What are the numbers shown in the results section meant to represent - they are geometric means at what age/time? How do you get a p-value to adjust for multiple measurements from the same animal - it should just be a correction to the standard errors. Some further detail/clarification in the manuscript should be provided.

To correct and improve our analyses and interpretation of the data we have now analysed the longitudinal part of the study by using repeated measures ANOVA (therefore not having to try adjusting for measurements at the different ages). This has allowed us to present estimated marginal means (or more precisely estimated geometric marginal means along with 95% confidence intervals, due to the logarithmic transformation of the data that was necessary prior to its analyses and the back transformation that was necessary prior to presentation). We have also performed more post-hoc testing to compare values from individual groups, rather than just relying on the overall p-values gained from ANOVAs or Kruskal Wallis ANOVA by ranks (this was not originally performed due to concerns about post-hoc and multiple testing). Such changes have necessitated a complete re-write of the Statistical Analysis part of the Methods section (pages 6-7), as well as the Methods part of the Summary (page 2), and the Urine Albumin Excretions and Albumin/Creatinine Ratios part of the Results section (pages 8-9).

What next?: Unable to decide on acceptance or rejection until the authors have responded to the major compulsory revisions

Level of interest: An article whose findings are important to those with closely related research interests

Quality of written English: Acceptable

Statistical review: No

Declaration of competing interests:  
I declare that I have no competing interests