Author's response to reviews

Title: Calcium deficiency assessment and biomarkers identification by an integrated urinary metabonomics analysis

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Author's response to reviews: see over
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Dear Editors:

On behalf of my co-authors, I am submitting the manuscript “Calcium deficiency assessment and biomarker identification by the integrated urinary metabonomics analysis” for publication in your honorable journal. We are very pleasure for that we can have this chance to submit our papers to such a leading international journal. Specially, we believe the paper may be of interest to the readers of your journal.

Calcium deficiency is a worldwide public health problem. Calcium nutrition status is currently assessed by a number of methods, such as epidemiological survey, calcium balance study, serum biochemical analysis and radiological examination; however, none of these is suitable for large-scale screening of calcium deficiency in a population or clinically accurate diagnosis of calcium deficiency at the early stage.

The molecular mechanism of calcium deficiency in detail remains somewhat elusive. Therefore, we investigated the metabolic changes and identified the biomarkers associated with calcium deficiency in a rat model by urinary metabonomics based on ultra performance liquid chromatography coupled with quadrupole time-of-flight tandem mass spectrometry and multivariate statistical analysis. The results suggest
that urinary metabolic profiling analysis could preliminarily distinguish between calcium deficient and non-deficient rats. Moreover, we established an integral strategy for discovering reliable biomarkers of calcium deficiency by combining the time-course analysis of discriminating metabolites in low-calcium diet experiment, repeated low-calcium diet experiment and calcium supplement experiment. The unbiased global urinary metabolic profiling combined with biomarkers can be applied not only in the accurate assessment of calcium deficiency at different stages but also for further unraveling the dynamic pathophysiological changes of calcium deficiency.

We found, for the first time, that purine metabolism and the Krebs cycle are possibly related to calcium metabolism. The implicated metabolic pathways, biological significances and the molecular mechanisms associated with calcium deficiency could be elucidated by identified biomarkers.

The urinary metabonomics analysis may lead to a more complete understanding of calcium deficiency. Significant correlations between dietary calcium intakes and urine citrate (Pearson Correlation, r=-0.426, p=0.001) or pseudouridine (Pearson Correlation, r=0.527, p=0.0001) were further confirmed in a population study. We anticipate in future that a noninvasive, sufficiently sensitive and specific diagnosis method for assessing calcium nutrition status accurately could be established based some of the identify biomarkers. In particular, the generic approach described here could be applied in the metabonomics studies of other nutrients deficiency, such as vitamin D.

We promise that the work described has not been submitted elsewhere for
publication, in whole or in part, and all the authors listed have reviewed the final version of the manuscript and approved it for publication.

Thank you very much for considering our manuscript for potential publication. We will be very grateful and appreciate to see our paper in your journal. I'm looking forward to hearing from your good news.

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Sincerely yours,

Maoqing Wang
Re: Manuscript Number 1482397859726363
“Calcium deficiency assessment and biomarkers identification by an integrated urinary metabonomics analysis”

Dear Editor:
Thank you very much for the review of our manuscript (Manuscript Number 1482397859726363 “Calcium deficiency assessment and biomarkers identification by an integrated urinary metabonomics analysis”). We have read the comments of the editor and three reviewers carefully. It is undisputable that all these comments are very valuable and precise for us to revise our manuscript.

We have revised this manuscript point-to-point as detailed below:

I. Response to the issues raised by Reviewer Connie Weaver

“The authors address as much needed assessment tool for assessing calcium status. Their design of deplete-replete is strong. Comparison to calcium balance is also strong.”

Thanks very much for your comments.

1. “The biggest concern is whether the findings can be translated into humans, especially on a wide variety of diets, medications, etc. The authors acknowledge this but can they offer any examples where similar findings are or are not translatable?”

Thank you very much for your valuable suggestion.

To verify the identified biomarkers associated with calcium deficiency in human and characterize the value of these biomarkers in clinical diagnosis, we designed and conducted a population epidemiology study. Seventy women (age: 50-64, 56.55±3.8) were recruited. The average dietary calcium intakes of included women were derived from three days of weighed dietary records. The 24h urine samples of all subjects were collected. The normalized peak areas of citric acid, pseudouridinie, and
pyrophosphoric acid in urine were measured by UPLC/Q-TOF MSMS. Significant correlations between dietary calcium intakes and urine citrate (Pearson Correlation, $r=-0.426$, $p=0.001$) or pseudouridine (Pearson Correlation, $r=0.527$, $p=0.0001$) were observed. However, there was no correlation between dietary calcium intake and urinary pyrophosphoric acid ($r=0.148$, $p=0.413$). The results further confirmed that the biomarkers (citrate and pseudouridine) which had also identified from rat model have the potential of being the diagnosis biomarkers in human.

We have added the population epidemiology study in the revised manuscript.

2. Discretionary Revisions

1) “Serum biochemical analyses are not good measures of calcium status. BMC by DXA would be better.”

Thanks very much for your suggestion.

I agree with that BMC by DXA was better than serum biochemical. The sensitivity and specificity of serum biochemistry testing are relatively low and unsatisfied. But, the radiological examination, can not give the diagnosis of calcium deficient at the early stage unless there is pathological injury to bone.

2) “Reference 10 for humans is not appropriate for rats.”

Thanks very much for your suggestion.

II. Response to the comments pointed out by Reviewer: Narattaphol Charoenphandhu

Wang et al. have developed a noninvasive, highly sensitive urinary metabonomics analysis to identify the biomarkers associated with calcium deficiency in male rats. Based on the ultra performance liquid chromatography coupled with Q-TOF tandem mass spectrometry, the authors reported several biomarkers possibly related to calcium deficiency. Despite a use of the state-of-the-art technique, there are major concerns as followed (one of which is related to further application in human).

Thanks very much for your comments.

1. “Since the ultimate goal of this noninvasive technique is to screen for calcium deficiency in human, why did the authors not perform an experiment in calcium-deficient subjects? Although an animal study could exclude some errors due to dietary calcium variation (as discussed by the authors) and age-dependent change in the intestinal calcium absorption, it is uncertain whether the biomarkers identified in the present study are the same as in human.”

“ar to confirm the potential application in human, the authors may simply ask healthy volunteers to provide their dietary calcium intake record and urine samples, assuming that many of them have low calcium intake (Refs. 3-5 in this manuscript). After measuring 2-3 candidate metabolites in the urine (e.g. pyrophosphoric acid, citric acid and uridine), the authors may observe a correlation between dietary calcium intake and each metabolite.”

Thanks very much for the precious suggestions.

Accurately, when we submitted this manuscript to your journal on line, we conducted a population epidemiology study to confirm the potential application of identified biomarkers from animal study.

As we know that, for the decreased level of estrogen and calcium loss, menopause or postmenopausal women are the main calcium-deficient subjects. Therefore, 70
women (age: 50-64, 56.55±3.8) were recruited for screening and verifying the calcium deficiency biomarkers in our revised manuscript.

The average dietary calcium intakes of included women were derived from three days of weighed dietary records. The 24h urine samples of all subjects were collected. The normalized peak areas of citric acid, pseudouridine, and pyrophosphoric acid in urine were measured by UPLC/Q-TOF MSMS. Significant correlations between dietary calcium intakes and urine citrate (Pearson Correlation, r=-0.426, p=0.001) or pseudouridine (Pearson Correlation, r=0.527, p=0.0001) were observed. However, there was no correlation between dietary calcium intake and urinary pyrophosphoric acid(r=0.148, p=0.413).The results further confirmed that the biomarkers (citrate and pseudouridine) which had also identified from rat model have the potential of being the diagnosis biomarkers in human.

We have added the population epidemiology study in the revised manuscript.

2. “Some statements in the introduction are not clear. For example, on page 3, “radiological changes, in general, cannot be observed unless there is pathological injury to bone”; what is the meaning of “pathological injury”? Do the authors mean fracture and vertebral compression? Indeed, radiological examination can reveal rickets and osteomalacia (non-injury lesion).”

Thanks very much for such a professional, accurate suggestion.

We have revised this sentence as follows:

Radiological changes or bone changes reflect a severe calcium deficiency which has already resulted in serious damage to the body. Therefore, radiological examination, in general, can not give the diagnosis of calcium deficient at the early stage unless there is pathological injury to bone (line 2-5, page 4).

3. “Is there a correlation between serum PTH levels and urinary phosphate excretion (if so, please describe statistics in the text)?
Thanks very much for this suggestion.

The level of urine creatinine and phosphorus were determined at week 12 using commercial Kits (Beijing Bioassay Technologies Co, Ltd and Wako Pure Chemical Industries, Ltd) by automatic biochemical analyzer. However, since there was no correlation between serum PTH levels and urinary phosphate excretion, we have not added this part in the revised manuscript.

Since 1,25(OH)2D3 can modulate FGF23 secretion, an increase in urinary phosphate excretion may not be explained solely by hyperparathyroidism. The serum levels of FGF23 are thus required to speculate the cellular mechanism of hyperphosphaturia.”

Thanks very much for such an excellent suggestion.

According to the suggestion of reviewer, the serum levels of FGF23 in LCG and NCG were measured by FGF23 ELISA Kits (Uscn Life Science Inc., Wuhan, China.). The serum level of FGF23 in LCG was significant higher than that in NCG (p=0.002) (Table 1), which indicated that increased FGF23 played an important role in hyperparathyroidism.

4. “The authors should provide catalog number, inter-/intra-assay %CV, and detection limit for each commercial ELISA kits.”

I am sorry for missing this information in our manuscript. Thanks very much for your careful work. We have added this information in our revised manuscript as follows:

(1) Serum parathyroid hormone (PTH) (ELISA Kits from ALPCO Diagnostics, Salem, NH): catalog number: Alpco 31-IPTMS-E01 Mouse/Rat Intact PTH EIA (96 wells); inter-/intra-assay %CV: CV %< 6% and CV %< 8%; detection limit: 1.0
pg/ml.

(2) 1,25(OH)2D3 (ELISA Kit for 1,25-Dihydroxyvitamin D3, Uscn Life Science Inc., Wuhan, China): catalog number: E90467Ge; inter-/intra-assay %CV: CV %< 5% and CV %< 7%; detection limit: 4.1pg/ml.

(3) FGF23 (ELISA Kit for Fibroblast Growth Factor 23 (FGF23), Rattus norvegicus (Rat), Uscn Life Science Inc., Wuhan, China): catalog number: E90746Ra; inter-/intra-assay %CV: CV %< 5% and CV %< 8%; detection limit: 6.1pg/mL.

Minor Essential Revisions

1.” Please check and correct typographical errors, e.g., “can not” in the abstract.”

Thanks very much for your careful work.

We have checked and corrected the typographical errors in the revised manuscript.

2. Tables 1 and 2 are missing (not included in the main PDF file).

Thanks very much for your suggestion.

We are very sorry for missing these two important Tables. They have been added in our revised manuscript (pages 31-33).
III. Response to the issues raised by Oleg A. Mayboroda.

Manuscript of Wang at al “Calcium deficiency assessment and biomarkers identification by an integrated urinary metabonomics analysis” touches an iterating topic and includes a strong, carefully designed study. However, reviewer’s opinion is that the manuscript in its current status is not ready for a publication. Data analysis contains too many mistakes and a part on “Metabolic pathways” has little scientific value.

Introduction:
Page 3: Authors start building their argumentation for their study claiming that exiting methods of calcium deficiency assessment are not “suitable for large-scale screening of calcium deficiency in a population.” As an alternative MS-based metabolomics is offered. A strong method, but it is far from being cheap and still to experimental to be applied for large scale studies.

Thanks very much for your suggestion.
We considered this question seriously for a long time. The MS-based metabonomics study is not cheap and the biomarkers still being experimented at present. And, it will be the major challenge for the application of metabonomics in large scale. However, the biggest advantage of UPLC/Q-TOF MS/MS is short time, high throughput, and accurate for detecting early changes of metabolites, which is exactly the prerequisite of detecting method using in the large scale study. No current method (such as epidemiological survey, calcium balance study, serum biochemical examination and radiological examination) has these functions. Therefore, as the development of UPLC/Q-TOF MS/MS, the cost of metabonomics may be reduced, MS-based metabolomics has the potential to be the method for the diagnosis of calcium deficiency in large-scale population study. Furthermore, new kits may be developed based on the biomarkers identified by MS-based metabolomics. Therefore,
the purpose of this study is to identify the biomarkers of calcium deficiency by MS-based metabolomics. We anticipated that these biomarkers can be used by clinically accessible assays in the diagnosis of calcium deficiency at early stage or in large scale population screening.

Page 4: “Metabonomics is an emerging science as “the quantitative measurement of the global, dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or perturbations of whatever source”[17-19]” A quote used by authors cannot belong to all three references. An original quote (from ref 17) is as following: “Metabonomics is defined as the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification.

Thanks very much for your suggestion.
We have removed the ref 18-19 in the revised manuscript.

**Experimental Section:**
Page 7: “Acetonitrile as a blank solution was run every 5th samples and the urine samples in the analysis batch were injected in random order.”
Were samples analyzed in a single batch or in several? In there were several batches, how batch to batch reproducibility was estimated?

Thanks very much for your suggestion.
1. The urine samples were analyzed in two batches: experiment I and II.
2. As shown in **Additional file 3**, the batch to batch reproducibility was estimated.

**Results:**
Page 10: “As shown in Table 1…”
Sorry, but where is the mysterious Table 1?
We are very sorry for missing Table 1 and Table 2.
In fact, we have uploaded these 2 Tables on line to your journal. There must be something wrong with my computer. I failed to check it before submitting. We have added them in the revised manuscript (pages 31-33).

Page 10: “As shown in Supplementary Figure 1…."
As it is now, legend of Supplementary Figure 1 is surely uncompleted, needs editing.

Thanks very much for your suggestion.
The legend of Supplementary Figure 1 has been edited in the revised manuscript (Additional file 2).

Page 10: “Quality assessment of the metabonomics platform”
The use pooled samples as quality controls for estimation of the analytical variability is a good idea, but some important information is missing for PCA plot:
a) How much variance is covered by the first two principal components?
b) How many principal components were calculated?
c) Was the model explored with regard to injection order bias and day to day variations?

Thanks very much for your suggestion. We are sorry for missing this information.
We have added this information in the revised manuscript as follows:
a) R2X= 12.5%, Q2=10.3%
b) 30 components
c) The model has been explored with regard to injection order bias and day to day variations in detail (Additional file 3).

Page 11: “Because more than 5000 ions were detected in rat urine…”
Authors never say how many samples their analyzed in total.
If we take in the account 24 animals per group (experiment I), two groups and 12 time
points, we are arriving at 576 samples (BTW, above mentioned Supplementary Figure 1 contains significantly less samples). However, even for a study with roughly 600 samples, 5000 variables per sample will lead to highly overfitted models. Authors should reconsider their basic data processing routines and try some data reduction approaches.

Thanks very much for such a professional suggestion.

Experiment I: 394 urine samples (24 rats per group*2*4weeks+16 rats per group*2*4+8 rats per group*2*4=384);

Experiment II: 432 urine samples (12 rats per group*3*12=432).

Experiment I: 384 urine samples (24 rats per group*2*4weeks+16 rats per group*2*4+8 rats per group*2*4=384);

Experiment II: 432 urine samples (12 rats per group*3*12=432).

Experiment II: repeated low calcium experiment (12 rats per group*2*12=288)
We have considered the basic data processing routines carefully. The data reduction approaches have been used in the revised manuscript.

All data were handled according to the “80% rule”; thus, only the variables with values greater than zero presenting in at least 20% of each group were kept for the following analysis. After the data reduction was handled according to the “80% rule”, 2850 (experiment I) and 3042 variables (experiment II) were used for multivariate statistical analysis. The models PCA and PLD-DA were reanalyzed in the revised manuscript.

BTW, above mentioned Supplementary Figure 1 contains significantly less samples.

Thanks very much for your suggestion.

The two-dimensional PCA scores plots of urine samples and QC samples of experiment I, repeated low-calcium diet experiment, and calcium supplement experiment were all added in the revised manuscript (Additional file 3).

Page 11-13: An approach taken by authors for further analysis of their data is clearly suboptimal (if not to say wrong). It is reviewer’s advice to authors to withdraw this manuscript and reanalyze the data completely. The biggest mistake is to use PLS-DA modeling, which is not a method of choice for the analysis of the time dependent phenomena. It might be a good idea to use a simple PLS regression with time as a response variable or (since authors are devoted SIMCA users) batch analysis. A study design, especially experiment I, is almost perfect for this approach.
Thanks very much for such a professional suggestion.

We read your article (Metabolic Profiling of Accelerated Aging ERCC1d/- Mice, Journal of Proteome Research 2010, 9, 3680–3687) very carefully. The batch partial least squares (PLS) method was used for the analysis of dynamic and continuous alterations of urinary metabolites with time between NCG and LCG from week 1 to 12. Batch PLS scores plot (Figure 3 and Additional file 6 figure D) showed that while at the first week there was overlap between two groups along the first component, at 2-12 weeks LCG rats were completely distinct from NCG rats.

To investigate the dynamic and continuous alterations of urinary metabolites during the genes is and progression of calcium deficiency, the metabolic trajectory analysis by PCA (Figure 5 and Additional file 6 Figure F) was used for the global urinary metabolic profiling analysis of LCG from weeks 1–12 in the revised manuscript.

It appears that multivariate modeling is not the strongest part of the author’s expertise, they never show any numbers, neither variance covered by PCA models, nor goodness of fit/goodness of prediction for PLS-DA. They use supplementary figure 3 as demonstration of their model robustness; well, it is rather a demonstration of high possibility to obtain such a model by random combination of samples (please pay attention of the slop of green line!). Besides, it is advisable to show cross-validated score plot for PLS-DA model; it may have less impressive visual appearance, but reflects the quality of the model better.

Thanks very much for your suggestion.

The parameters associated with PCA models and PLS-DA models were all included in the revised manuscript.

The goodness of fit/goodness of prediction and cross-validated score plot for PLS-DA model were shown in Additional file 4 and Additional file 6 Figure C.

Finally, using VIP cut-off 1.0 with heavily very fitted data is certainly a mistake.
Thanks very much for your suggestion.
We are sorry for choosing an unwise cut-off value (1.0). The VIP cut-off 1.5 was used in the revised manuscript.

We have addressed all the suggestions of the three reviewers. All the revised and added content have been marked by red in the revised manuscript. Undoubtedly, the incorporation of the three reviewers’ comments has improved the quality and accuracy of this manuscript. Although it is not easy to write in a second language, we will try our best to revise this manuscript until it is qualified for publication. Thanks very much.

Sincerely yours,

Wang Maoqing