Author's response to reviews

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

Authors:

Maoqing Wang (wang_maoqing@yahoo.com)
Xue Yang (yaya_orange2005@yahoo.com.cn)
Fan Wang (Yifan.701@163.com)
Ran Li (yo_yo1983@126.com)
Hua Ning (ninghua188@126.com)
Lixin Na (nalixin2003@yahoo.com.cn)
Yifan Huang (yzzhyf@hotmail.com)
Yue Song (wolingdong@126.com)
Liyan Liu (yanziliu2100@163.com)
Hongzhi Pan (panhongzhi@163.com)
Qiuju Zhang (zqj81129@sina.com)
Lijun Fan (fanlijun1229@sina.com)
Ying Li (liying_helen@163.com)
Changhao Sun (Sun2002changhao@yahoo.com)

Version: 3 Date: 20 November 2012

Author's response to reviews: see over
Dear Ursula D'Souza, PhD
Senior Editor,

Thank you for the review of our manuscript (Manuscript Number 1704763993787749 “Calcium deficiency assessment and biomarkers identification by an integrated urinary metabonomics analysis”). We have addressed the comments of the editor and three reviewers carefully and edited the format of our paper to conform to the journal style. The addressed comments in the revised manuscript were highlighted in a red color. Your and the reviewers’ comments were extremely helpful in enabling us to improve our paper.

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

I. Response to the issues raised by Narattaphol Charoenphandhu

Minor Essential Revisions
1. The authors should provide catalog number, inter-/intra-assay %CV, and detection limit for each commercial ELISA kits on Page 7. (the authors have mentioned in the cover letter that they have added this information in the revised manuscript; however I did not find it in the Methods section).

Thank you for your suggestion. We apologize for not including this information. The catalog number, inter-/intra-assay %CV, and detection limits for three commercial ELISA kits have been provided in the Methods section on page 7 in our revised manuscript (R2 version).

   Serum parathyroid hormone (PTH, 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), rat fibroblast growth factor 23 (FGF23, catalog number:
E90746Ra; inter-/intra-assay %CV: CV %< 5% and CV %< 8%; detection limit: 6.1 pg/mL) and 1,25(OH)₂D₃ (catalog number: E90467Ge; inter-/intra-assay %CV: CV %< 5% and CV %< 7%; detection limit: 4.1 pg/ml) were determined by ELISA method (ELISA Kits from ALPCO Diagnostics, Salem, NH, and Uscn Life Science Inc., Wuhan, China).

2. Additional file 10 (correlation between dietary calcium intakes and urine pseudouridine and citrate) should be presented as Figure 9.

Thank you for your suggestion.

Additional file 10 (correlation between dietary calcium intakes and urine pseudouridine and citrate) has been added as Figure 8 in our revised manuscript (R2 version). The original Figure 8 was presented as Figure 9 on page 18 in our revised manuscript (R2 version).

II. Response to the issues raised by Oleg A. Mayboroda

New version shows that authors have spent some time trying to improve manuscript. However, in my opinion manuscript is not reaching the standards required for the publication BMC Medicine.

The most serious problem is an apparent disturbance between the data presented and the conclusions made. Let’s consider the conclusion section of the manuscript:

“We demonstrated for the first time that the unbiased global urinary metabolic profiling combined with biomarkers provided a robust method for diagnosis of calcium deficiency in the different stages, especially for the early stage. The pathophysiological changes and molecular mechanisms of calcium deficiency were elucidated by some identified biomarkers. Based on the further verification of more biomarkers in human, we anticipate that the biomarkers identified in our study can be used for population screening and clinical diagnosis of calcium deficiency in the future.”

Strictly speaking this is a combination of the overstatements. What authors do
demonstrate with this work is that using LC-MS based metabolomics workflow they can identify the differences between two groups on experimental animals. No more, no less. An attempt to present this data as a robust diagnostics (!) is an enormous overstatement (to say the least).

Thank you for providing professional and valuable suggestions. We apologize for this fault. Finding “a robust method for diagnosis of calcium deficiency in the different stages” is the highest aim of our study. However, as you have indicated, we have not achieved this objective based on the work of this study.

We have revised this section as follows:

“We identified for the first time the reliable biomarkers of calcium deficiency in male rat by the time-course analysis of discriminating metabolites in a low calcium diet experiment, repeating the low calcium diet experiment and performing a calcium supplement experiment. The identified biomarkers give new insights into the pathophysiological changes and molecular mechanisms of calcium deficiency. The correlations between calcium intake and two biomarkers provide the potential for further assessment and elucidation of the metabolic responses of calcium deficiency in humans. Based on further verification of these two biomarkers in large population, we anticipate that these two biomarkers might be used for population screening and clinical diagnosis of calcium deficiency in the future.”

However, we sincerely want to ask for your advices. If the manuscript requires further revisions, please do not hesitate to tell us. We are pleased to revise our manuscript until it attains the required standards for publication by BMC Medicine. Thank you.

In details:
1) Authors made multiple claims on translational value of their study aiming of using the discovered “biomarkers” for the human situation. The authors themselves (page 11) and one of the reviewers both have indicated that the postmenopausal women represent the core of the risk population (human validation cohort at least was built on this assumption), the same time male rats used for all experiments. How do authors explain their selection of the animals? Knowing that gender is one of the strongest confounders in the metabolomics how do they envision translation of their result to the human situation?

Thank you for the excellent and professional advice. We considered your opinion and discussed it carefully.

The main aim of our study was to identify the reliable biomarkers for accurately assessing calcium nutrition status and exploring the metabolic alterations associated with the genesis and progression of calcium deficiency in a low calcium diet rat model. However, in metabonomics studies, a major challenge is how to distinguish the reliable biomarkers that are closely associated with the genesis and progress of diseases from those that are unrelated but are altered. To solve this problem, we developed an integrated screening method that consists of the following steps. First, the discriminating variables with regular and reasonable changing trends were selected as potential biomarkers by time-course analysis. Second, the repeatability of biomarkers is crucial for their identification and biological interpretation in metabonomics study. To verify the repeatability of biomarkers, the same urinary metabolic profiling and time-course analysis of discriminating variables were applied in a repeated low calcium experiment. Third, a calcium supplement experiment based on the low calcium experiment was used to further confirm the above biomarkers. To achieve these objectives, it needs large amount of work and time. Therefore, we only chose male rats in the animal model in this
study.

It’s a pity that the female rat were not selected as the subjects, thus, we apologized for that we can not explain the confounders of gender in the metabolomics based on our experiments now. It is also a limitation of this study. We have no enough time to repeat all the experiments of female rate within the limited revised time. But, we plane to add these experiments in our future study.

Our ultimate goal is establish a sensitive and noninvasive tool for accurately assessing calcium nutrition status and identify biomarkers that can elucidate the mechanisms underlying calcium deficiency in humans. To achieve this goal, two steps were designed as follows. Firstly, we planed to identify the reliable biomarkers of calcium deficiency by low calcium diet rat model. Secondly, we plane to verify the potential of practical applications of the reliable biomarkers of calcium deficiency from our animal models in humans in the future. In the first version of our manuscript, we only showed the results of metabonomics study of low calcium diet male rats.

Considering the biomarkers identified from animals cannot be used in humans directly, two reviewers suggested that we translate these findings into humans.

“The biggest concern is whether the findings can be translated into humans, especially on a wide variety of diets, medications, etc.” “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.”

Therefore, to provide the potential application of the biomarkers of calcium deficiency from our animal experiment in humans, we added a population research in the revised
manuscript (R1 version).

Fortunately, our group is performing a survey of the calcium intake and nutrition status of postmenopausal women in Harbin city. Postmenopausal women have been reported to be the core of the risk population. To investigate the correlations between calcium intake and the biomarkers of calcium deficiency, we chose postmenopausal women as the subjects of our population research.

As shown in our results, significant correlations were found between these two biomarkers (pseudouridine: Pearson correlation, \( r=0.527, p=0.0001 \) and citrate: Pearson correlation, \( r=-0.426, p=0.001 \)) and calcium intake. Fortunately, although there are species and gender differences, these two calcium deficiency biomarkers were cross-validated in both male rats and middle-aged women. These data suggested that these two biomarkers are not affected by species and gender; they may be the specific markers of calcium deficiency.

However, this study in postmenopausal women about the correlations between calcium intake and two biomarkers only provides a potential application of these two biomarkers in humans. To achieve our ultimate goal, a large and well-designed population study (including male and female subjects) with the confounders being controlled is urgently needed. For now, we are performing a large and well-designed population study. We anticipate that we can establish a noninvasive and accurate diagnostic method for assessing calcium deficiency status by using these biomarkers, which is also the highest aim of our study.

So, we expressed our opinion above. Maybe, these are some mistakes. We sincerely want to ask for your advices. Thanks very much again.
2) 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”. Is this sentence complete?

Thank you for your suggestion. It has been revised on page 4 as follows:

Metabonomics involving “the quantitative measurement of the global, dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or perturbations of whatever source” is an emerging science.

3) Page 4. “high mass spectral signal-to-noise ratios” – it is very confusing, I only can guess what authors mean.

Thank you for your suggestion. “High mass spectral signal-to-noise ratios” was replaced by “high sensitivity” on page 4 in the revised manuscript (R2 version).

4) Page 5. “Urine is essentially the body’s liquid waste repository.”

Thank you for your suggestion. This sentence was revised on page 5 as follows:

“As a rule, the best biofluid to study nutrient intake or to identify biomarkers is urine. Urine is essentially the body’s liquid waste repository, and any endogenous or exogenous metabolites that are not needed or present in excess may be found in the urine.”

5) Page 13. It is not clear why authors decided to include in their multivariate analysis only 8 animals form each group having 24 animals per group. Their explanation “For clear illustrative purpose” is not sufficient.

Thank you for your suggestion.
There were 24 rats per group from weeks 1-4. At the end of weeks 4 and 8, 8 rats were selected randomly from each group and sacrificed. Therefore, only 8 rats were maintained throughout weeks 1-12. For investigating the dynamic metabolic alterations and changing trend during weeks 1-12, urine samples of these 8 rats were selected for multivariate analysis. However, this selection of samples might not reveal reliable changing trend between two groups.

Therefore, according to the suggestion of reviewer 3, all rat urine samples (384) were included for the multivariate analysis in the revised manuscript (R2 version) (as shown in revised Figures 2, 3 and 5).

Next, authors used this selection for PCA and PLS-DA modeling. The values for $R^2_X$ (0.779) and $Q^2$ (0.753) point towards a strong model. Logically enough, authors provide a supplementary figure (File 4) showing the results of cross-validation. However, the initial values on the plot (blue and green dots on the right) are significantly higher than indicated in the text. Thus, the cross-validation data do not belong to the discussed model!

Thank you for your diligent work.

We have rechecked and reanalyzed the data carefully. Using the first two components of the PLS-DA model, we determined the following: $R^2_X$ =0.779 and $Q^2$=0.753. We provided these values for $R^2_X$ (0.779) and $Q^2$ (0.753) in the main document in the revised manuscript (R1 version).

Using the first six components of the PLS-DA model, we determined the following: $R^2_X$ =0.928, $Q^2$=0.854. Additional file 4 showed these values for $R^2_X$ =0.928, $Q^2$=0.854 in the revised manuscript (R1 version).
We apologize for the inconsistency on the values for R2X and Q2 between in the main document and Additional file 4.

The details of data are as follows:

<table>
<thead>
<tr>
<th>Component</th>
<th>R2Y(cum)</th>
<th>Q2(cum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Comp[1]</td>
<td>0.653354</td>
<td>0.633245</td>
</tr>
<tr>
<td>Comp[2]</td>
<td>0.779033</td>
<td>0.75266</td>
</tr>
<tr>
<td>Comp[3]</td>
<td>0.856303</td>
<td>0.804292</td>
</tr>
<tr>
<td>Comp[4]</td>
<td>0.892837</td>
<td>0.829876</td>
</tr>
<tr>
<td>Comp[5]</td>
<td>0.910879</td>
<td>0.83779</td>
</tr>
<tr>
<td>Comp[6]</td>
<td>0.928185</td>
<td>0.854326</td>
</tr>
</tbody>
</table>
In the revised manuscript (R2 version), these values for R2X and Q2 in the main document and Additional file 4 both show $R^2 = 0.826$ and $Q^2 = 0.687$ for the first five components of the PLS-DA model.

6) **Figure 3.** Authors give the following interpretation of the figure: “Batch PLS scoresplot (Figure 3) 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 my option it is more correct to say that despite clear observed separation tendency the groups were quite different already at the first week of experiment.

Thank you for your careful work.
However, we were unable to understand your comment: “despite clear observed separation tendency the groups were quite different already at the first week of experiment”.
Do you mean, “Except for the clear separation tendency, the groups were already quite different at the first week of experiment”?

Figure 3 has been re-edited in our revised manuscript (R2 version). Batch PLS scores plot (Figure 3) showed that excluding the first week, the clear separation tendency between the two groups was observed from weeks 2-12.

7) **Page 14, Figures 3 and 4.** Figure 3 and Figure 4 represent two different ways to communicate the same message. However, it is clearly that there is some inconsistency between the figures. If Figure 3 shows that after the 4th week the profiles remain more or less stable, Figure 4 has to prove that “metabolic distance” between the groups is increasing. The most evident expiation of this phenomenon is the biased selection of samples included in PCA model showing 9 weeks data (Figure 4C).

Thank you for such an excellent suggestion.

We apologize for the inconsistency between the figures. This may have been due to only 8 of the rats being maintained throughout weeks 1-12 and selected for the batch PLS analysis.

As you suggested, all of the rat urine samples (384) have been included for the batch PLS analysis of Figure 3 to reduce the inconsistency between the figures in the revised manuscript. The revised Figure 3 has been added. As shown in Figure 3 and 4, the trend change of Figure 3 was almost consistent with that of Figure 4 on page 14.

8) **Metabolic Pathways, page 17-20.** To my opinion the entire section no scientific value at all, at least when it is a part of the result section. Authors **provide no experimental evidence** for their “pathway elucidation” and in a way show notorious lack of understanding of human biochemistry and physiology. Once more, as a **part of discussion** and in much more condensed form this part has some right to existence, as a
part of result section it must be excluded from the manuscript.

Thank you for this excellent suggestion.
In accordance with the suggestion of the reviewer, the part of Metabolic Pathways in the Results section was presented as a part of the Discussion section (from paragraph 2 on page 21 to paragraph 1 on page 23) in the revised manuscript (R2 version).

9) Page 20. “Calcium deficiency is reversible when found at an early stage. But, without proper intervention, it can develop to an irreversible stage, such as rickets and osteoporosis.” I absolutely disagree with this statement. To reduce etiology of osteoporosis to a simple calcium deficiency is an unacceptable oversimplification.

Thank you for this detailed suggestion.
As you suggested, osteoporosis is a recognized complication due to several factors and disorders. The most important risk factors for osteoporosis are advanced age (in both men and women) and female sex. Calcium deficiency alone cannot lead to osteoporosis. Therefore, osteoporosis was deleted from this sentence.
We have revised this sentence on page 18 in the revised manuscript (R2 version) as follows:
Calcium deficiency is reversible when diagnosed at an early stage. However, without proper intervention, calcium deficiency can develop to an irreversible stage, such as rickets.

We have read all of the suggestions of the two reviewers carefully and revised our manuscript point-by-point. We are most grateful to the two reviewers, and to Professor Oleg A. Mayboroda, whose comments and suggestions have enabled us to greatly improve our paper.

In accordance with the reviewers’ suggestion, this manuscript has been edited by Edanz.
If this manuscript requires further revisions, please do not hesitate to tell us. We are grateful and appreciate the opportunity to revise this manuscript until it has qualified for publication in your journal.

I am looking forward to hearing from you soon.

Sincerely yours,

Wang Maoqing