Author's response to reviews

Title: Bone resorption and environmental exposure to cadmium in children

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Author's response to reviews: see over
Dear Editor,

Thank you very much for having given us the opportunity to revise our manuscript: “Bone resorption and environmental exposure to cadmium in children” (MS 7304657605778198).

We carefully addressed all editorial comments and the comments of the reviewers. We checked carefully the discrepancies between text and tables and corrected this in the new version. A point by point reply to the comments of the referees is given below.

Reviewer 1: Maryka H Bhattacharyya

The authors present the results of a study showing a significant direct correlation between urine cadmium concentration (measure of Cd exposure) and urine calcium and DPD concentrations (measures of bone status) in 160 children 8-12 years of age living in Lahore, Pakistan. Although such studies have been conducted in adults, in particular postmenopausal women, a study in this age group is new. One strength of the study is the large number of children and the clarity of the results. A weakness is the lack of attention to detail applied by the authors regarding presentation of methods and consistency of values reported at different points in the manuscript (see specific comments below).

Specific comments:

1. The authors should give the range in times-of-day during which their spot urine samples were collected, because they indicate that time-of-day affects urine DPD concentration, and because they account for time-of-day in their data analyses. Were most samples collected at one time of day?

   In the revised version we added the time of the day (page 6; first paragraph, line 1 measurements in urine). In addendum 1 the distribution of the times of day is given. In addition, to exclude circadian influence on our data we include time of the day in our regression model which did not alter the reported associations (result section, page 8, and paragraph 2), see addendum. The number of children was equally distributed over time.

2. The authors need to indicate if care was taken to account for calcium precipitation when their urine samples were frozen. If not, their urine calcium values are low. Calcium precipitation – e.g., 23% of total calcium for a sample in mid-range of normal -- has been documented, even when urine is frozen at 20 °C for one overnight (doi:10.1152/ajprenal.90736.2008). One approach is to acidify the urine before aliquots are taken for calcium analysis. However, the described protocol indicates that a 500 µl aliquot of the thawed urine sample was taken, and then the aliquot was diluted with acid in preparation for analysis. Neglecting calcium precipitation may not have been a critical flaw [because precipitate amount was proportional to urine Ca concentration (above reference)], but this aspect of study protocol should be addressed.

   In the revised version we stated that the urine samples were not acidified by EDTA (page 7; second paragraph, line 3 measurements in urine). Because Ca precipitation is proportional to the urinary Ca concentration, it is unlikely that this has biased our results. Our low Ca concentrations are in line with recent findings from Berglund, et al (Environ Res. 2011, online) in children. Low urinary Ca excretion in children can be attributed to rapid growth.

3. The authors should give the limit of detection for cadmium in urine and other characteristics of the Cd analyses, e.g., intra- and inter-assay coefficients of variation. As is, one COV value is given, but it is not clear which assay this value refers to (probably DPD ELISA). The current approach lacks detail that is needed for this report to stand on its own.
In the revised manuscript we clarified that the coefficient of variation referred to the ELISA measures for deoxypyridinoline excretion. In addition, we added the inter-assay coefficient of variation for Cd was <3% for certified internal control (Recipe, ClinCheck control level I and II). The limit for detection (LOD) and the limit of quantification (LOQ) for cadmium were 0.015 µg/l and 0.044 µg/l respectively. We added this to the revised method section on page 7, line 7.

4. Table 1 and Figure 1. The authors need to address the point that results for urine Cd concentration (Cd-U) in Table 1 appear to disagree with those presented in Figure 1. The median Cd-U value in Table 1 is 0.54 µg Cd/g creatinine, and the 95th percentile value is 0.58 µg Cd/g creatinine. Those results mean that only eight values of the 160 should be >0.58 µg Cd/g creatinine (upper 5%). The latter spread from Table 1 is very different from the spread of Cd-U values shown in Figures 1a and 1b, where many more than eight values are > 0.58 µg Cd/g creatinine. Maybe the 95th percentile value for Cd-U is wrong in Table 1.

We corrected the discrepancies between table 1 and figure 1.

5. Table 1. The value of this important dataset would be enhanced by adding two columns to Table 1 that give individual datasets for the males vs. females, in addition to the combined column already presented. For other age ranges, data for males vs. females have been broken out in published reports. Expansion of this table will allow other investigators to compare gender-based differences across ages, including the new age range presented here.

We formatted table 1 as suggested by the referee.

6. Table 2. The ‘b’ superscript on the second ‘calcium’ biomarker line appears to be in error. It is not clear how a correlation between urinary calcium and urinary cadmium can be adjusted for urinary calcium, as indicated by the superscript. Probably the added adjustment for the calcium biomarker line was for DPD.

We have corrected as suggested.

7. Results 1st para and Table 2. The authors need to address the point that results in the text differ from those in Table 2. The text states that a doubling of urinary Cd corresponds to an increase in urine DPD of 1.86 ng/g creatinine (95% CI: 1.52 to 2.29; p: <0.0001), taking into account urine Ca, along with gender, age, height, weight and socioeconomic class. In Table 2, the values for this same regression are similar but not identical (1.80: 1.44 to 2.22; p: <0.0001).

We corrected.

8. Discussion, 2nd para. Comparison of urine cadmium concentrations in Pakistan (this study) vs. Europe should use values with the same units – either µg Cd/g creatinine or µg Cd/L urine. As is, the authors use different units for the values they compare. Granted the numerical values for these two units are often similar, but readers will not in general know that.

We harmonized units as suggested by the referee.

9. Figures 1a and 1b. It is suggested that the data-points be changed so they indentify gender (e.g., by different shapes), to allow the reader to visualize the relationships for the girls vs. boys in the combined dataset. From gender-based differences in means reported here, the girls should be clustered high and the boys low for both Cd U and DPD.

The referee makes an important suggestion to improve our visual presentation by gender specific shapes. We changed figure 1 as suggested. The revised figure clearly shows that our results were not biased by gender clusters of the studied variables.

10. Abstract vs. Body of Manuscript: The expressions of results in the abstract need to be changed to match those given in the body of the manuscript. For example, the 71% increase in DPD for a doubling of Cd U given in the abstract is expressed in Table 2 as regression
We carefully checked all numbers in results, abstract and tables and corrected where necessary.

11. The authors have the unusual opportunity to evaluate specificity of the cadmium results they report, because they used ICP-MS to analyze many metals in urine. I suggest providing an evaluation of lead in urine vs. bone indicators also, to investigate specificity regarding the cadmium results. These two metals co-exist in nature and both have an effect on bone. Other investigators, though not many, have done this and found a statistically significant correlation for cadmium but not lead. These results strengthen the argument of cause and effect for an experiment where only correlations can be made.

As suggested by the referee, we have checked the association of urinary DPD and urinary lead. Apparently, no association between urinary DPD and urinary lead was present in our cohort of children (r=0.03; p=0.6). This might indicate that lead does not promote bone demineralization. On the other hand it might be anticipated that stored lead in bones enters the circulation after demineralization by cadmium, but we did not observe this. In this regard we should also be aware that urinary lead is not the ideal biomarker for lead exposure which is blood lead. However, we did not draw blood samples in the current study; therefore we believe that we do not have the best opportunity to focus on lead.

12. Discussion, General: Inclusion of a discussion is suggested regarding why the measures of bone demineralization (urine calcium) and bone resorption (urine DPD) were not themselves significantly related to one another. These markers are both individually related to urine Cd and are each used as indicators of Cd-induced bone loss. The hypothesis seems to follow that, when cadmium increases bone demineralization (urine Ca), it would also increase release of collagen cross-links (urine DPD), giving reason to expect a relationship between the two bone markers, but this is not the case.

We were also surprised that no significant correlation exists in our cohort of children between urinary DPD and urinary calcium. Urinary DPD correlates with indices of resorption in bone biopsies (Roux G et al. Bone 1995;17:153-6). It is a specific marker of bone resorption while urinary calcium might apart from bone demineralization also been influenced to some extent by calcium intake and by tubular kidney function, as well as by rapid growth relevant in this specific age group.

Reviewer 2: Yasushi Suwazono

The aim of this study was to investigate the association between markers of bone demineralization [urinary calcium (U-Ca) and deoxypyridinoline (U-DPD) excretion] and urinary cadmium (U-Cd) excretion (as an index of lifetime body burden). However, the measured U-DPD and U-Ca seemed to be much lower than the normal levels reported in previous studies. The association between U-Ca, U-DPD and U-Cd suggested in this child population seems to be so interesting and valuable that the present measurement should be carefully checked and revised adequately. Those estimates make a very important contribution to the international discussion on the risk assessment of environmental cadmium exposure in the general child population. Therefore, I recommend the authors to resubmit after careful revision of this manuscript.

Major compulsory revisions

1. Abstract: The authors noted that “A doubling of urinary Cd was associated with a 71% ($p<0.0001$) increase in urinary DPD and, a 33% ($p=0.0006$) increase in urinary Ca.” However, these values were different from those in table 2.

We carefully checked all numbers in results, abstract and tables and corrected where necessary.
2. Methods: Please clarify whether the measured U-DPD was ‘free’ U-DPD or ‘total’ U-DPD.

   We added in the revised manuscript that we measured free DPD (page 7, second paragraph, line 3).

3. Methods: Gender difference existed for U-Cd and U-DPD. I recommend the authors to divide the subjects into two groups according to gender, in order to make easier to interpret the results and to apply the results to actual population.

   The referee makes an important suggestion to allow better interpretation of potential gender induced bias, therefore, we stratified table 1 by gender. In addition, as suggested by referee 1 (point 9) we give gender specific shapes in revised figure 1.

4. Results (page 8, lines 17-18): The authors noted “each doubling of urinary Cd was associated with an increase in urinary DPD of 1.86 ng/g creatinine (95% CI: 1.52 to 2.29; p: <0.0001).” These values were different from those in table 2.

   We carefully checked all numbers in results, abstract and tables and corrected where needed.

5. Results (page 8, lines 17-18), Table 2: The authors reported that doubling of U-Cd may yield very small increase (1.86 ng/g creatinine) in U-DPD compared to the median level of 337 ng/g creatinine. As the authors estimated regression coefficient for each doubling of U-Cd, I guess the authors adopted 2 as the base of log-transformation of U-Cd, U-Ca and U-DPD. Based on this assumption, these regression coefficients can be presented as the anti-logarithm of these values and interpreted as doubling of U-Cd was associated with increase in U-DPD by 3.32(2^1.86) times. Another interpretation is that the presented values such as 1.86 were already converted as the anti-logarithm of obtained regression coefficients. The unit (ng/g creatinine and mg/g creatinine) should be deleted to avoid misinterpretation.

   The values were indeed retransformed to the linear scale; we added this in revised statistical method section (page 8 line 4).

6. Table 1: The median of U-Ca (3.56 mg/g creatinine) seemed to be very small compared to the mean U-Ca (0.155 g/g creatinine=155mg/g creatinine) in primary school children in north of Iran. (http://www.ncbi.nlm.nih.gov/pubmed/16388158) The measurement of U-Ca should be checked carefully and revised adequately.

   Our results might be partly (about 25%) influenced by freezer induced precipitation of Ca. Because Ca precipitation is proportional to the urinary Ca concentration, it is unlikely that this has biased our results. Our low Ca concentrations are in line with recent findings from Berglund, et al (Environ Res. 2011, online) in children. Low urinary Ca excretion in children can be attributed to rapid growth.

7. Table 1: The median of U-DPD (337ng/g creatinine) seemed to be very small compared to the median of free U-DPD (14-21 nmol/mmol creatinine) in primary school children in United Kingdom. (http://www.ncbi.nlm.nih.gov/pubmed/10086947) Based on the molecular weight of DPD (413g/mol) and creatinine (113g/mol), 337ng/g creatinine of U-DPD corresponds to 0.092 nmol/mmol creatinine. The measurement of U-DPD should be checked carefully and revised adequately. Furthermore, the unit should be converted to “nmol/mmol creatinine” for U-DPD.

   According to the suggestions of the referee we have checked the units of DPD carefully and changed where necessary. In the revised version we converted the units to mmol (also for other measurements).

8. Table 2 “Calcium, mg/g crtb”: In this model, U-Ca was adjusted to estimate regression coefficient for U-Ca. This is incorrect calculation. This line should be deleted.
In secondary analysis, urinary calcium was adjusted for deoxypyridinoline in urine. We clarified this in the revised version.

9. Discussion (page 10, line 3): The authors should add at least one citation for “Children take up Cd more readily than adults due to lower iron stores.”

We referred in the revised version to Berglund et al. Gender and age differences in mixed metal exposure and urinary excretion. Envirion Res 2011. We also extend the discussion on the iron-cadmium relation (page 10, paragraph 2).

Reviewer 3: Frank Thevenod

This study is very important for the field. It is the first study demonstrating a possible toxic impact of low level environmental Cd exposure on bone occurring already in children and has at least three major consequences: 1) DPD may represent an early biomarker of environmental exposure to low levels of Cd, particularly at a stage where the kidney is not affected; 2) Cd-induced bone damage may occur early in life and impact on subsequent osteoporosis in adulthood, particularly in older people; 3) More experimental studies in vitro and in vivo describing direct osteotoxic effects of Cd on bone are necessary to complement this epidemiological study. Refs 24 and 25 are simply not sufficient and published in vitro studies are not convincing enough.

Major compulsory revisions

Though it is assumed that the children population studied was healthy and had no kidney damage, is there any evidence from the literature that the incidence of renal damage is similar in children from Pakistan compared to children from Western countries?

We do not have indications from the literature for higher renal damage in children from Pakistan compared with children from western countries.

Minor essential revisions

First paragraph of results section should read “(Figure 1 a & b)”. Changed as suggested.

Discretionary revisions

The higher urinary DPD and Cd concentrations in girls is striking, but is not well discussed. The comparison to the situation in mature women, who have lower iron stores due to menstrual bleeding and subsequent intestinal upregulation of divalent metal transporter-1, suggests that a significant proportion of the girls had already reached puberty and went through menstrual cycles. Though this may not have been properly investigated for cultural reasons, this could be commented on. Otherwise, alternative speculations may arise, such as different roles of boys and girls, the latter being more exposed to household chores (cleaning, gardening) and hence to Cd containing dust, etc.

The gender based issue of higher DPD and Cd is important. In the revised version we address this point in more detail to exclude that our results were biased by residual confounding for gender. We present gender specific shapes in revised figure 1 (see also point 9, referee 1). Our revised figure shows that urinary Cd and DPD levels were higher in women but that the association between urinary DPD and Cd was present in both boys and girls. We extended the discussion on iron and cadmium uptake (2 of the discussion; page: 10).
We hope that with these clarifications our revised manuscript will be accepted for publication.
We are looking forward hearing from you.

Sincerely Yours,

Muhammad Sughis, Joris Penders, Vincent Haufroid, Benoit Nemery and Tim Nawrot
Addendum 1

![Histogram of TOV density](image)