PEER REVIEW HISTORY

ARTICLE DETAILS

TITLE (PROVISIONAL)	A Cross-Sectional Study Measuring Vanadium and Chromium Levels in Paediatric CKD Patients
AUTHORS	Filler, Guido; Kobrzynski, Marta; Sidhu, Hargun; Belostotsky, Vladimir; Huang, Shih-Han; McIntyre, CW; Yang, Liju

VERSION 1 - REVIEW

REVIEWER	Ryszard Grenda The Children's Memorial Health Institute, Warsaw, Poland
REVIEW RETURNED	27-Oct-2016

GENERAL COMMENTS	Very interesting study, indicating the risk of enviromental pollution and accumulation of specific microelements in children with reduced renal function. The best view on this problem would be achieved if the authors could present the results of evaluation of healthy control group, including peers living in the same area, as patients. Anyway, presented results are of substantial value. Clinically - the major problem is real clinical relevance of increased concentration of vanadium and chromium in these patients. Data from animal (experimental) investigations show, that chronic overload with vanadium and chromium results in functional and structural damage in proximal and distal tubules. If so, one might expect that patients with eGFR about 50 mL/min/1.73m2 could present some symptoms of proximal or distal tubulopathy. The deserves comment in the discussion. The experimental data show also, that older subjects (experimental animals) are more prone the toxic effect of microelements, so it would be interesting whether the age (apart form eGFR) was correlated with concentration of vanadium and chromium. Also, in cases in whom renal graft biopsy was performed (for any classic clinical indications) – the information on any signs of tubular damage in pathomorphological picture (or absence of such findings) would be appreciated (in discussion).

REVIEWER	Frederick Kaskel Albert Einstein College of Medicine Children's Hospital at Montefiore Bronx, NY USA
REVIEW RETURNED	11-Nov-2016

GENERAL COMMENTS	Is there any seasonal variation in the data, i.e., summer vs winter exposures?

REVIEWER	Darcy Weidemann Children's Mercy Hospital University of Missouri - Kansas City USA
REVIEW RETURNED	17-Nov-2016

GENERAL COMMENTS	Overall I think this is an interesting and useful study to better understand and define exposure to various trace elements which is woefully understudied, particularly in pediatric CKD patients. As there are few modifiable risk factors for CKD progression, I think further investigation into exposure to various metals deserves further attention and applaud the authors on addressing this important issue. My specific comments related to the text is below: Abstract 1. Line 24: Why is your primary outcome plasma zinc levels? Why do your secondary outcomes include plasma chromium, copper, and molybdenum? Why should I even care about these elements? Also in line 7 you define chromium short-hand as Cr and vanadium as V, so to save space you could use Cr and V in all subsequent text. I would suggest that Plasma V and plasma Cr are your primary outcomes. Secondary explorations could be other trace elements including X, Y and Z. I understand this is an ancillary substudy off of another study, although really the primary objective in THIS study is plasma V and plasma Cr. 2. Lines 27-33: For clarity of reading, I would suggest consistency in how you present V and Cr results. What is the median (IQR) V level? You state the median Cr but not V level. Introduction 1. Would more clearly define if you are interested in looking at pediatric CKD patients not on dialysis or comparing CKD to non-ESRD patients. Patients and Methods 1. Figure 1 – I think this figure is very confusing. Would specifically clarify if dialysis-dependent or not in your inclusion/exclusion criteria. Did you also include CKD 1 (i.e. those with “normal” GFRs)? I think you should specifically state CKD 1-5, including dialysis dependent (or not). I think if you include dialysis-dependent patients I think you should specifically state this N. If you are going to include the N of those who were excluded based on age (n=3) then would also include those who were excluded based on renal transplant and eGFR > 90. Then, it’s not really “Excluded (n=1)” , it is n=3 excluded and n=1 who declined to participate. I would suggest making this box “Declined to participate (n=1)”. Also, would suggest keeping your denominator consistent, i.e. patients not measurements. Then divide by Subjects with single measurements (n=17) and subjects with repeat (?multiple) measurements, (n=19). I am also not clear on the difference between multiple versus repeat measurements. How far apart were the measurements in subjects who had repeat values?

	2. How does fluid or food intake impact the results? I could see how fluid intake could impact urinary levels of heavy metals (based on concentration) but am not sure how it would affect plasma levels of trace elements. 3. Why did you use the modified Schwartz formula for your inclusion criteria but the Filler formula for your outcomes? 4. Would specify “anthropomorphic data” and “blood parameters” which is a little vague. You don’t actually present any anthropomorphic data in table 1 anyways so not sure you need to include it, unless you are referring to height and serum creatinine (to calculate GFR). 5. Were V, Cr, and eGFR levels left or right-skewed? You could also try log-transforming for your analyses which can often lead to more normal distribution approximation if you want to do any regression analysis in the future. 6. Wilcoxon Signed Rank test is appropriate for comparing two related/paired samples, I’m not sure it is appropriate to compare C levels with the 97.5% of a reference range. What reference range of “normal” did you use for V and Cr levels, and are they pediatric specific? 7. I think the additional incorporation of postal codes and heat maps in the drinking water is a very nice and elegant addition to the study. Results 1. I know that V and Cr levels aren’t normally distributed. I’m assuming that they are right-skewed but a figure demonstrating the overall distribution of the values for V and Cr would be a useful addition to the paper, especially since one of your major points of the paper is that V and Cr are elevated in children with CKD. It would also be useful to superimpose the “normal” reference ranges onto this diagram. 2. Table 2 – I am concerned that the “normal” reference ranges are based on what appears to be a single-institution lab memorandum, which appear to be based on only 124 healthy adult individuals and I am not sure this is based on a peer-reviewed published data. I also feel that the authors need to explain why they chose to use these specific reference. Would suggest referring to the Handbook on the Toxicology of Metals (ed, Nordberg, Nordberg, Fowler, and Friberg 2007) where they do have some pediatric reference ranges which are different than what is used by the authors (i.e. children vanadium levels 0.024-0.226 ug/L which is a much higher “normal” value than what is cited by the author, there’s another adult reference range which is 0.09-0.75 ug/L). It is false to label this table as “pediatric reference intervals’ as they are based on adult data, and I also think this needs to be acknowledged as a limitation in the discussion. 3. Page 9 line 21 “above the reference interval for healthy children and adults” is incorrect, as above these are unpublished reference intervals based on 124 “healthy” adults. Would remove the phrase “children and”

	4. Page 10 line 6-8 “comparing these distributions leads us to believe that our findings are the result of both environmental factors and impaired kidney function” really belongs in the discussion, not in results. 5. I’m not sure that figures 3 and 4 add much to the paper. I would suggest keeping this as text and adding a figure which demonstrates the overall distribution of V and Cr levels in your population (see point 2). You could also consider combining Figures 2 and 4 into one figure with a part A and part B, as I think it is nice to look at them side by side. Discussion 1. Not a major point but I think the adverse consequences of Cr and V are probably better discussed in the introduction section. Also would be important to distinguish between acute toxicities (i.e. extremely high exposures) versus chronic low-level exposure, which is harder to prove adverse consequences for many of the metals including V and Cr. Also I would also comment on the carcinogenicity of chromium which is another adverse consequence worth mentioning. 2. Limitations – suggest adding that robust, pediatric-specific reference ranges for “normal healthy” children are lacking, and that your study relied on unpublished reference data from an adult population.

VERSION 1 – AUTHOR RESPONSE

Reviewer 1

Very interesting study, indicating the risk of environmental pollution and accumulation of specific microelements in children with reduced renal function.

1. The best view on this problem would be achieved if the authors could present the results of evaluation of healthy control group, including peers living in the same area, as patients. Anyway, presented results are of substantial value.

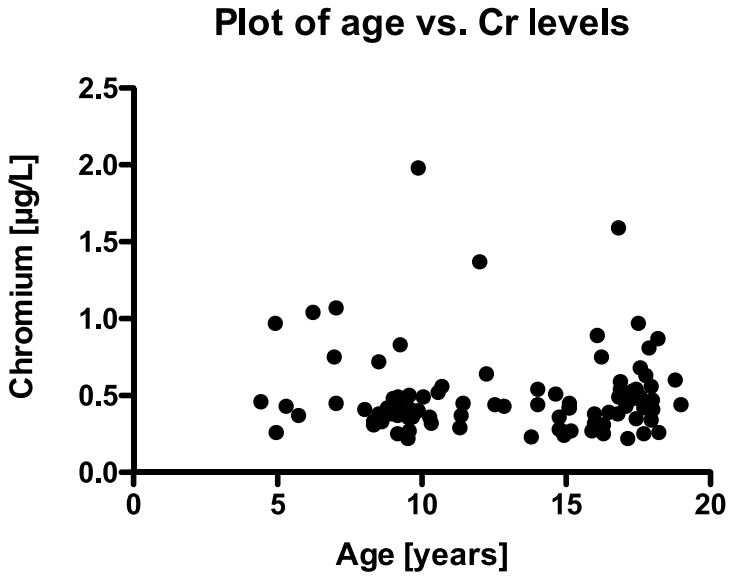
   The reviewer is correct that this would augment the data, however unfortunately we do not have access to an age- and gender-matched control group; our comparator was the use of very recent reference values for the trace elements, which were taken from a healthy population in our catchment area. Certainly siblings without CKD would have been an ideal control group, but the study was not designed with a control group in mind. As we cannot accommodate this request, no changes were made to the text.

2. Clinically - the major problem is real clinical relevance of increased concentration of vanadium and chromium in these patients. Data from animal (experimental) investigations show, that chronic overload with vanadium and chromium results in functional and structural damage in proximal and distal tubules. If so, one might expect that patients with eGFR about 50 mL/min/1.73m^2 could present some symptoms of proximal or distal tubulopathy. This deserves comment in the discussion.

When designing the study, we did not expect this finding and we did not collect markers of tubular injury such as urinary beta-2-microglobulin/creatinine ratio. We agree with the reviewer that this would be very interesting. This is especially important as there is growing evidence that Cr(IV)-related tubulotoxicity could successfully be reversed with selenium supplementation. The authors are aware of a recent study in Chemosphere that nicely demonstrated this in a chicken model. Similar findings were demonstrated in a study of Cr(IV)-induced nephrotoxicity in adult rats (Ecotoxicol Environ Saf. 2010 May;73(4):671-8. doi: 10.1016/j.ecoenv.2009.10.002.) We also agree with the reviewer that the relationship between Cr levels and tubular dysfunction should be evaluated in studies such as the CKiD study. We added the following sentences to the end of the discussion regarding the effect of Cr on humans. This new section reads as follows:

Additionally, the tubulotoxicity of Cr, in particular, may influence the progression of CKD. Although we did not examine the tubulotoxicity found in our patient cohort, there is often mixed tubular and glomerular proteinuria in CKD patients and proximal tubular dysfunction in CKD.[42] Some recent research in animal models has suggested that selenium supplementation could reverse some of the tubular damage.[43]

3. The experimental data show also, that older subjects (experimental animals) are more prone the toxic effect of microelements, so it would be interesting whether the age (apart form eGFR) was correlated with concentration of vanadium and chromium.
We took this into consideration and tested for a correlation between V, Cr, and age; there was no significant correlation between the trace element level and age. We have included the figure of this analysis for Cr below, but elected against including this information in the text as to not confuse the reader. Since age was not important in the univariate analysis, we felt that this information should be omitted.



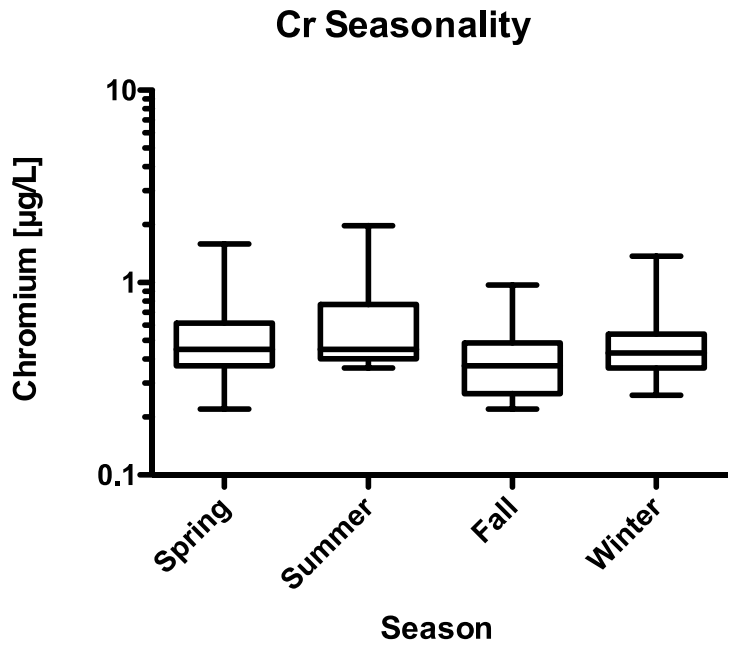
Also, in cases in whom renal graft biopsy was performed (for any classic clinical indications) – the information on any signs of tubular damage in pathomorphological picture (or absence of such findings) would be appreciated (in discussion).

We agree with the reviewer, but did not prospectively gather this information in our study. We do not perform surveillance biopsies in our centre, therefore many patients may not have had renal biopsies. We do, however, agree with the reviewer in that this is indeed an interesting question for an ancillary study; we intend on addressing this question in a future manuscript.

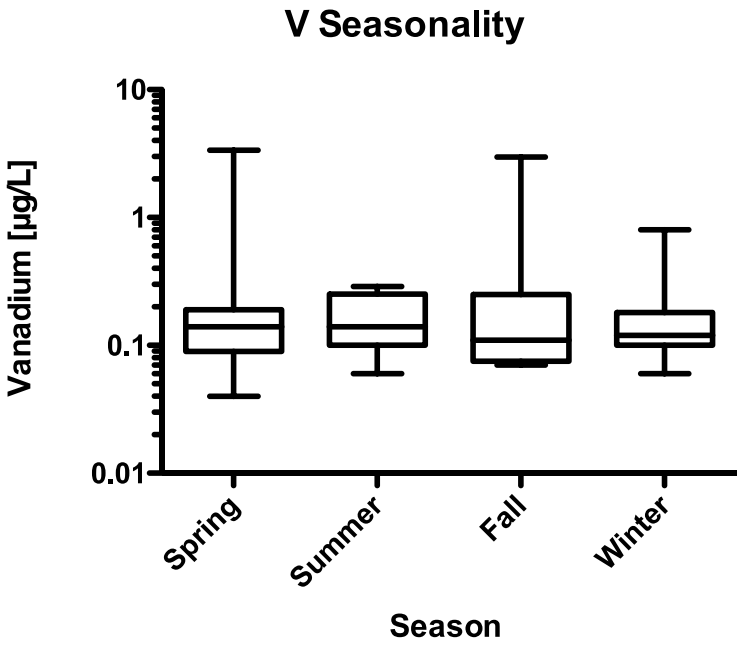
Reviewer 2

1. Is there any seasonal variation in the data, i.e., summer vs winter exposures?

This is an interesting question and was not something we had considered. To answer the question, we defined Spring as March-May, Summer as June-Aug, Fall as Sep-Nov, and Winter as Dec-Feb. We then plotted the Cr and V values and used the Kruskal-Wallis test to assess seasonal variability. There was no variability between the seasons. For Cr, the Kruskal-Wallis test p-value was 0.1303. The Cr values are plotted below:



For V, the Kruskal-Wallis test p-value was 0.8883. The plot for V seasonality is given below:



We added one sentence to the Results section to address both this point as well as the third point raised by Reviewer #1. This added sentence reads as follows:

Neither V nor Cr levels were associated with age or season.

Reviewer 3

Overall I think this is an interesting and useful study to better understand and define exposure to various trace elements which is woefully understudied, particularly in pediatric CKD patients. As there are few modifiable risk factors for CKD progression, I think further investigation into exposure to various metals deserves further attention and applaud the authors on addressing this important issue.

We sincerely thank the reviewer for her kind words, and wholeheartedly agree that this is understudied and warrants further investigation.

My specific comments related to the text is below:

Abstract

1. Line 24: Why is your primary outcome plasma zinc levels? Why do your secondary outcomes include plasma chromium, copper, and molybdenum? Why should I even care about these elements? Also in line 7 you define chromium short-hand as Cr and vanadium as V, so to save space you could use Cr and V in all subsequent text. I would suggest that Plasma V and plasma Cr are your primary outcomes. Secondary explorations could be other trace elements including X, Y and Z. I understand this is an ancillary substudy off of another study, although really the primary objective in THIS study is plasma V and plasma Cr.

   The Reviewer is correct that the primary outcome for this specific study was the measurement of plasma trace elements. This study was an ancillary study to the zinc study, hence the confusion on our end. The information about the zinc study is irrelevant to the reader and should not be included. The molybdenum results are subject to a different paper, and we did study all the other trace elements but have not analysed them yet. We modified the "Primary Outcome Measures" section of the Abstract as follows:

   Primary and Secondary Outcome Measures: Primary outcomes: Plasma Cr and V. Secondary outcomes: Age, season, CysC, CysC eGFR, and Cr and V levels in environmental water.

   These specific metals were part of a trace element panel at our core laboratory (see http://www.lhsc.on.ca/lab/metals/). We chose plasma rather than whole blood or erythrocytes because it required a smaller blood sample, which was an important consideration in a paediatric study.

   We also removed the long-form versions of V and Cr from subsequent text.

2. Lines 27-33: For clarity of reading, I would suggest consistency in how you present V and Cr results. What is the median (IQR) V level? You state the median Cr but not V level.

   The Reviewer is correct – the median (IQR) V level was added as follows:

The median V level was 0.12 µg/L (0.09, 0.18).

We also added the number of patients with high Cr levels as follows:

… 34 had at least one set of Cr levels above the published reference interval.

Introduction

3. Would more clearly define if you are interested in looking at pediatric CKD patients not on dialysis or comparing CKD to non-ESRD patients.

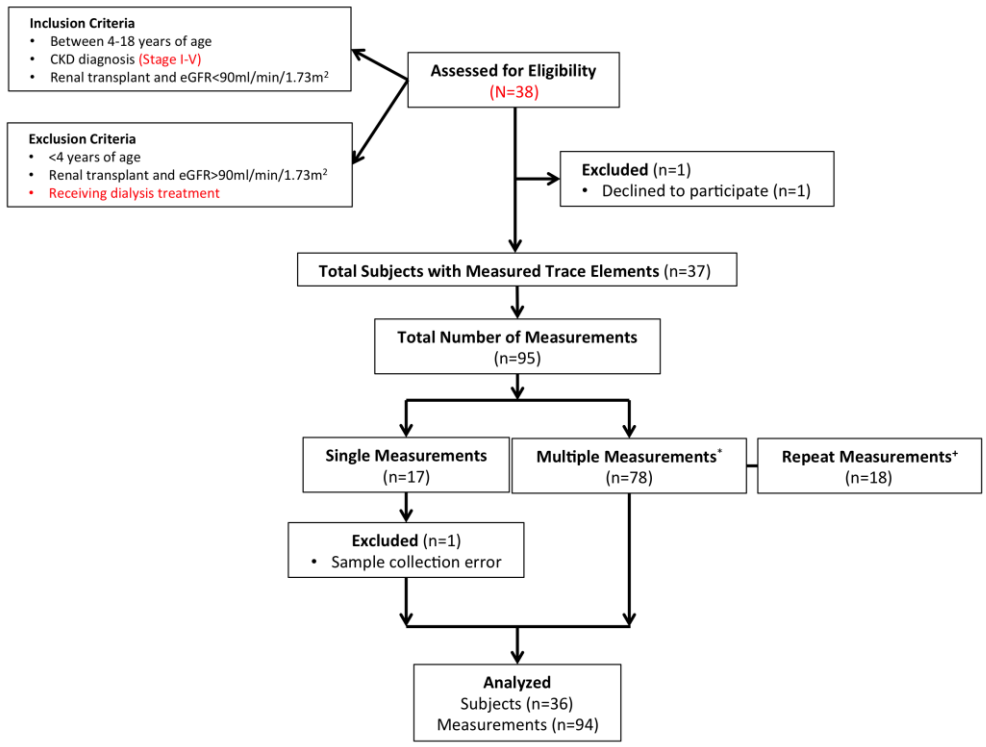
   As per the Reviewer's request, we clarified that we were interested in looking at paediatric CKD patients who were not receiving dialysis treatments. The text was modified as follows:

   Although data in paediatric CKD patients are elusive, we hypothesized that similar to dialysis patients, we would find elevated V and Cr levels in paediatric CKD patients who were not receiving dialysis treatments.

Patients and Methods

4. Figure 1 – I think this figure is very confusing. Would specifically clarify if dialysis-dependent or not in your inclusion/exclusion criteria. Did you also include CKD 1 (i.e. those with "normal" GFRs)?

   We realize that this was unclear in the figure and have modified Figure 1 accordingly. As per your comment below, we removed the n=3 excluded due to their young age (<4 years old). Additions to the figure are highlighted in red. Of note, we excluded dialysis patients because they may have had their V and Cr levels altered from the dialysis water.



*Multiple measurements: measurements taken over multiple clinic visits
+Repeat measurements: measurements analyzed on the same day and from the same blood sample

I think you should specifically state CKD 1-5, including dialysis dependent (or not).

The reviewer is correct. We added the following sentence to the study population section:

Patients with Stage 1 (eGFR> 90 mL/min/1.73 m^2) to Stage 5 (<15 mL/min/1.73 m^2) CKD were included in the study. Patients receiving dialysis treatments were excluded from the study because the dialysis water could further affect the patients' V and Cr levels.

I think if you include dialysis-dependent patients I think you should specifically state this N.

We excluded dialysis patients. We do however have a few measurements in dialysis patients. In one patient with a cystatin C >6.80 mg/L (eGFR<3 mL/min/1.73 m^2), the V level was 58.2 ug/L, and the Cr level was 54.4 ug/L. Both are the highest levels the undersigned has ever seen. She is anuric and on haemodialysis, and we have no dialysis water measurements at this point in time. There are just too many variables to include with dialysis patients, and PD patients may be rather different than HD patients. We are planning a prospective study that will include adult dialysis patients.

If you are going to include the N of those who were excluded based on age (n=3) then would also include those who were excluded based on renal transplant and eGFR > 90. Then, it's not really "Excluded (n=1)" , it is n=3 excluded and n=1 who declined to participate. I would suggest making this box "Declined to participate (n=1)". Also, would suggest keeping your denominator consistent, i.e. patients not measurements. Then divide by Subjects with single measurements (n=17) and subjects with repeat (?multiple) measurements, (n=19). I am also not clear on the difference between multiple versus repeat measurements.

We modified Figure 1 to accommodate these suggestions and believe it is now much clearer (see above).

How far apart were the measurements in subjects who had repeat values?

Repeat measurements were taken on the same day as a result of some confusion with the electronic medical records system. Multiple measurements were measurements that were taken at the next visit. The median interval between visits was 3 months. Since this is the incorrect statistical approach to this question, that result would provide the reader with the wrong impression; the statistically correct approach is a repeated measures analysis (see below). We therefore did not elect to include this information.

As stated above, the difference between the multiple vs. repeat measurements was when a patient received the plasma panel at separate clinic visits (i.e. over the course of several months) vs. if the patient received 2 plasma panels at the same clinic (i.e. in the same day). In order to limit any confusion, we added a sentence to the text clarify this point:

A repeated measures analysis was conducted to determine whether the results of repeated measurements (from the same day) affected the original results.

5. How does fluid or food intake impact the results? I could see how fluid intake could impact urinary levels of heavy metals (based on concentration) but am not sure how it would affect plasma levels of trace elements.

   As the Reviewer previously stated, drinking water may be a major source of V and Cr. Patients with CKD often have significant polyuria and subsequently develop polydipsia to prevent dehydration. With CKD, there is a slow decline of residual nephron endowment, resulting in a reduced ability to concentrate or dilute the urine. The fluid intake of a patient at Stage 3 or 4 of CKD may be as high as 8 l per day. V and Cr are eliminated based on the total nephron endowment, and since the ability to excrete worsens with a lower eGFR, they will accumulate in plasma while the patient's capacity to excrete them via urine is exhausted.

6. Why did you use the modified Schwartz formula for your inclusion criteria but the Filler formula for your outcomes?

This was a part of our study protocol. At the time that these data were collected, cystatin C turn-around-time was one week. Cystatin C eGFR is, however, superior to the bedside Schwartz formula.[1] Since March of 2016, the cystatin C turnaround time is 1 hour and had we performed the study today, we could have changed the inclusion criteria. This CysC turnaround time only applies at one centre and not at the other study site. No changes were made to the text.

7. Would specify "anthropomorphic data" and "blood parameters" which is a little vague. You don't actually present any anthropomorphic data in table 1 anyways so not sure you need to include it, unless you are referring to height and serum creatinine (to calculate GFR).

   The Reviewer is correct. We modified the text to clarify this point. We also opted to specify that we extracted creatinine and cystatin C from the EMR. This section now reads as follows:

   Anthropomorphic data (patient height measured by stadiometer, necessary to calculate the Schwartz eGFR), the first three digits of patients' postal codes, and creatinine and cystatin C were collected from our electronic chart program, PowerChart (Cerner).

8. Were V, Cr, and eGFR levels left or right-skewed? You could also try log-transforming for your analyses which can often lead to more normal distribution approximation if you want to do any regression analysis in the future.

   V and Cr were log normally distributed (see our response to Reviewer 2). The same applied for CysC, but eGFR was left-skewed. It is not always clear that log transformation normalizes the distribution, hence the use of non-parametric tests such as the Kruskal-Wallis analysis named above in response to Reviewer 2. No additional changes were made to the text.

9. Wilcoxon Signed Rank test is appropriate for comparing two related/paired samples, I'm not sure it is appropriate to compare C levels with the 97.5% of a reference range. What reference range of "normal" did you use for V and Cr levels, and are they pediatric specific?

   The reference intervals are not paediatric but are specific to our region, please see our response to Reviewer #1. This point was also specified in Table 2.

   Please see the comments on the Wilcoxon Signed Rank test from GraphPad Prism (the program that we used for our analysis) below. It appears that we used the appropriate test.

Interpreting results: Wilcoxon signed rank test

The Wilcoxon signed rank test is a nonparametric test that compares the median of a column of numbers against a hypothetical median. Don't confuse it with the Wilcoxon matched pairs test which compares medians of two paired groups).

The nonparametric Wilcoxon signed rank test compares the median of a single column of numbers against a hypothetical median.
Interpreting the P value
The P value answers this question:
If the data were sampled from a population with a median equal to the hypothetical value you entered, what is the chance of randomly selecting N data points and finding a median as far (or further) from the hypothetical value as observed here?

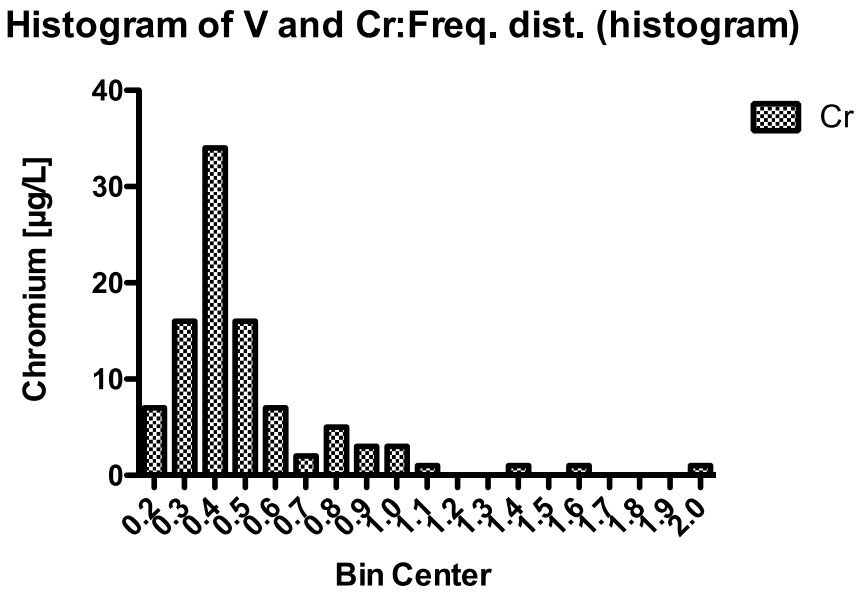
10. I think the additional incorporation of postal codes and heat maps in the drinking water is a very nice and elegant addition to the study.

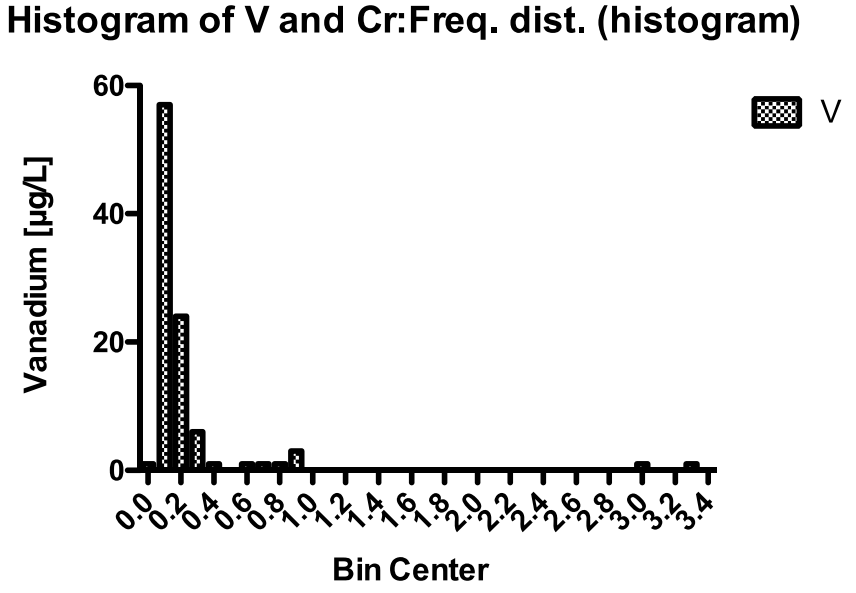
    We appreciate the Reviewer's kind comment.

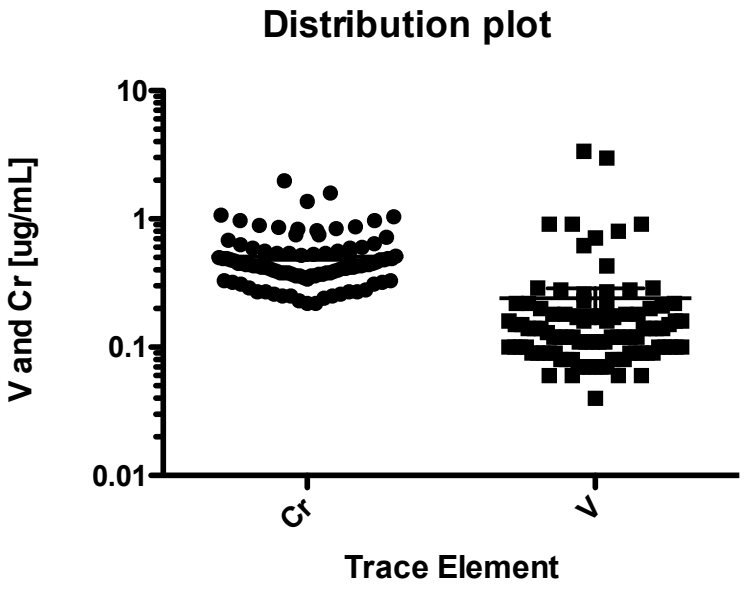
Results

11. I know that V and Cr levels aren't normally distributed. I'm assuming that they are right-skewed but a figure demonstrating the overall distribution of the values for V and Cr would be a useful addition to the paper, especially since one of your major points of the paper is that V and Cr are elevated in children with CKD. It would also be useful to superimpose the "normal" reference ranges onto this diagram.

    To address your request, we created two frequency distribution plots for V and Cr and have added them below. Unfortunately, journal requirements limit the number of figures we can include in our manuscript. Additionally, the main message of our study is that the trace elements accumulate with worsening eGFR. We therefore elected against including this additional data. Of note, should this interest the reader, the information can be extracted from the existing figures. No changes were made to the text.







12. Table 2 – I am concerned that the "normal" reference ranges are based on what appears to be a single-institution lab memorandum, which appear to be based on only 124 healthy adult individuals and I am not sure this is based on a peer-reviewed published data. I also feel that the authors need to explain why they chose to use these specific reference. Would suggest referring to the Handbook on the Toxicology of Metals (ed, Nordberg, Nordberg, Fowler, and Friberg 2007) where they do have some pediatric reference ranges which are different than what is used by the authors (i.e. children vanadium levels 0.024-0.226 ug/L which is a much higher "normal" value than what is cited by the author, there's another adult reference range which is 0.09-0.75 ug/L). It is false to label this table as "pediatric reference intervals' as they are based on adult data, and I also think this needs to be acknowledged as a limitation in the discussion.

    The reference intervals depend on the equipment used to measure the trace elements. Although the Reviewer is correct in that the results have not been published and were based on a single institution lab memorandum, our laboratory uses a very sensitive and interference-free mass spectrometry and they determined these normal values based on blood samples from healthy adults using the same method that they employ for children.
    We very recently obtained data from 26 patients that were referred in (but did not have their creatinine measured) over the last 2 years from across the country. Most values were from Hospitals in Common (or actually from Calgary). The average Cr level was 0.13 +/- 0.08 µg/mL, which was significantly lower than that of our patients. For V, it was 0.086 +/- 0.048 µg/mL, which is again significantly lower than the 0.12 that we had for the median of the patient V levels. Although we don't have detailed laboratory results for these patients, based on these data we can argue that the V and Cr plasma levels of children without renal impairment are most likely not higher than those seen in healthy adults and can therefore infer that levels in health children are not higher than in healthy adults.

13. Page 9 line 21 "above the reference interval for healthy children and adults" is incorrect, as above these are unpublished reference intervals based on 124 "healthy" adults. Would remove the phrase "children and"

    The author is correct, we removed the part of the sentence that read "children and". The phrase now reads as follows:

    Thirty-four patients had at least one Cr level that was above the reference interval for healthy adults (77 of 94 tests [82%]).

We also re-wrote the title and legend of Table 2 to ensure that this point was made clear to readers.

14. Page 10 line 6-8 “comparing these distributions leads us to believe that our findings are the result of both environmental factors and impaired kidney function” really belongs in the discussion, not in results.

    The author is correct. We took out that sentence and inserted the following sentence into the Discussion section:

    Since some groupings of patients corresponded to the areas of high V and Cr and others did not, this leads us to believe that our findings are the result of both environmental factors and impaired kidney function.

15. I’m not sure that figures 3 and 4 add much to the paper. I would suggest keeping this as text and adding a figure which demonstrates the overall distribution of V and Cr levels in your population (see point 2). You could also consider combining Figures 2 and 4 into one figure with a part A and part B, as I think it is nice to look at them side by side.

We aren’t sure what the Reviewer means - did the Reviewer mean to write that Figures 3 and 5 are not necessary? We disagree, and as outlined by Reviewer two, there is interest in longitudinal data. Our statistician gave us expert advice and strongly suggested using a repeated measures analysis, which we had done. Clinicians are interested in knowing how and if these values change over time, and while there was a trend towards higher values at the last measurement, this trend was not significant. Since we did not have a prospective longitudinal component in our study, we felt it was important to share the longitudinal data that we had available to us. We agree that this could be better studied in a large prospective cohort such as the CKiD study, and we have every intention of doing so once these pilot data are published. We elected to leave the figures as they are.

Discussion

16. Not a major point but I think the adverse consequences of Cr and V are probably better discussed in the introduction section. Also would be important to distinguish between acute toxicities (i.e. extremely high exposures) versus chronic low-level exposure, which is harder to prove adverse consequences for many of the metals including V and Cr. Also I would also comment on the carcinogenicity of chromium which is another adverse consequence worth mentioning.

We appreciate the Reviewer’s point regarding the placement of the information about the toxicity of V and Cr. It is true that little is known about these trace elements within the medical community, and that placing this information within the introduction rather than within the conclusion would make the paper more compelling to read in full. We have therefore moved the section regarding their toxicity into the Introduction.
We appreciate the Reviewer’s comment concerning this section, and following a second review of the literature, we re-organized and re-wrote the section on health effects and believe it is now much clearer, and that it highlights the data that is most pertinent to our study. First, we only included human data, since this is most relevant to our population. Second, we clarified which effects were associated with acute vs. chronic exposure, and we clarified the route of exposure.
Unfortunately, there is no published literature regarding the effect of chronic oral V exposure on humans. There are only 2 published studies examining the effect of intermediate oral exposure to V; the populations were small (21 subjects between the two studies), and the longest period of exposure was 12 weeks. We therefore opted to include chronic inhalation data. The new section reads as follows:

The danger V and particularly Cr pose to our health are made clear when the serious systemic effects associated with either acute or chronic exposure to either element in humans are explored in greater depth.
Acute oral exposure to V can cause mild gastrointestinal irritation such as stomach cramps,

mild diarrhoea, and nausea.[7] Although there are no studies examining the effect of chronic oral exposure to V on human health, chronic exposure to V through inhalation may cause neurocognitive deficits and may impair neurobehavioral abilities, symptoms which are regularly seen in paediatric CKD patients.[18] Chronic exposure to V through inhalation has also been shown to cause immunological effects such as significant decreases in lymphocyte stimulation and an increase in the incidence of viral and bacterial respiratory infections in children, a vulnerable population.[16] Another study examining the health effects of chronically inhaling V found a significant association between V and systolic blood pressure and pulse pressure in an elderly population.[17]
Cr appears even more toxic; acute oral exposure has been linked to (i) death, and at lethal doses respiratory effects such as pleural effusions, pulmonary oedema, bronchitis, and bronchopneumonia; (ii) cardiovascular effects such as cardiopulmonary arrest, cardiac arrest, and a drop in cardiac output, heart rate and blood pressure; (iii) gastrointestinal haemorrhage and necrosis, abdominal pain and vomiting; (iv) haematological effects such as inhibited coagulation; (v) hepatic effects such as the development of jaundice, fatty degeneration, hepatic necrosis, and increased bilirubin, serum lactic dehydrogenase, alanine and aspartate aminotransferase, and γ-glutamyl transferase; and (vi) renal effects such as renal failure characterized by proteinuria, haematuria and anuria, renal necrosis, necrosis and swelling of renal tubules, oliguria, and destruction of the tubular epithelium of the kidneys, highly elevated serum creatinine and blood urea nitrogen; and (vii) metabolic acidosis.[8] Two studies in particular have shown the effects of chronic oral exposure to Cr(VI). Both studies are exceptionally relevant to our study and consisted of populations who 1had Cr(VI)-contaminated drinking water. The first study, from an area near a ferrochromium production plant in the Liaoning Province in China, found an association between contaminated water and gastrointestinal effects such as oral ulcers, diarrhoea, abdominal pain, indigestion, and m kincreased incidence of lung and stomach cancer.[8 19] The second study, from an area of Greece with elevated Cr(VI) in the public drinking water, found significantly higher standardized mortality ratios for primary liver, lung, kidney, and genitourinary cancer.[8 20]

We appreciate the Reviewer's comment regarding the carcinogenicity of Cr – we feel this is important as well. We have two sentences that address this point in the manuscript (in the introduction), which read as follows:

"The IARC has classified Cr as carcinogenic. Inhalation has been shown to cause lung cancer in humans, and exposure has been shown to cause tumours in the stomach, intestinal tract, and lungs in animals.[2]"

When re-organizing and re-writing the section on health effects (now in the Introduction), we also added data regarding the oncological effects of chronic oral exposure to Cr(VI).

17. Limitations – suggest adding that robust, pediatric-specific reference ranges for "normal healthy" children are lacking, and that your study relied on unpublished reference data from an adult population.

We agree with the Reviewer in that this is a limitation, and have included the following sentence in our section of Limitations:

Additionally, although the reference intervals that we used were specific to our catchment area and to the equipment used to measure plasma trace elements at our site, they are based on a cohort of adults and have not been published.

1. Filler G, Yasin A, Medeiros M. Methods of assessing renal function. *Pediatr Nephrol* 2014;29(2):183-92. doi: 10.1007/s00467-013-2426-7 [published Online First: 2013/02/19]
2. Services USDoHaH. Toxicological Profile for Chromium United States: United States Government; September 2012 [Available from: http://www.atsdr.cdc.gov/ToxProfiles/tp7.pdf accessed July 11 2016.

VERSION 2 – REVIEW

REVIEWER	Ryszard Grenda The Children's Memorial Health Institute Poland
REVIEW RETURNED	10-Jan-2017

GENERAL COMMENTS	The revised version meets all previous comments and suggestions

REVIEWER	Darcy Weidemann University of Missouri - Kansas City United States of America
REVIEW RETURNED	25-Jan-2017

GENERAL COMMENTS	This is an excellent revision of the initial manuscript. Specifically, the study population is better defined, the introduction more clearly describes adverse outcomes of vanadium/chromium, and the appropriate limitations (i.e. lack of pediatric-specific reference ranges) are acknowledged in the discussion. I think this is a useful study which attempts to better understand and define exposure to trace elements in pediatric CKD patients, which is woefully understudied in our field, and would like to see it published.