Title: Predictors of serum dioxin levels among adolescent boys in Chapaevsk, Russia: A cross-sectional study.

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Version: 2 Date: 24 February 2005

Author's response to reviews: see over
February 24, 2005

David Ozonoff, MD
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Dear Dr. Ozonoff,

Enclosed is a copy of our revised manuscript entitled “Predictors of serum dioxin levels among adolescent boys in Chapaevsk, Russia: A cross-sectional pilot study”. Please consider this manuscript for publication in Environmental Health: A Global Access Science Source.

We thank the reviewers for their helpful comments on our manuscript. We welcome the opportunity to address each of the reviewers’ comments and suggestions.

Reviewer #1: Linda Birnbaum, USEPA

Review of MS# 8603706375292680: Hauser et al.;, Predictors of serum dioxin levels

Comment 1: This paper is scientifically sound, but I’m not sure of how important it is. Chapaevsk, Russia is a small city which is highly contaminated with many toxic chemicals, both organic and metallic. While there is contamination by PCBs and some dioxin-like compounds, the heavy metal contamination is potentially much more severe. Other exposure studies carried out in this area have indicated heavy contamination from cadmium and arsenic, among other inorganics. This study clearly shows that there is very little TCDD contamination. In fact, the dioxin-like compounds seem dominated by polychlorinated dibenzofurans (PCDFs) and biphenyls (PCBs), suggesting that the key contamination is due to PCBs and potentially pentachlorophenol.

Response: Among this small sample of adolescents, the detection limits (DLs) were high (ranging from 1.5 to 16.5 pg/g lipid for 2,3,7,8-TCDD), so in 28 out of 30 boys, TCDD was below the DL. However, given the lack of data on dioxin levels in children and the potential for differential exposure risk factors in children compared with adults, we feel the study contributes to the literature and to our understanding of predictors of dioxin exposure. In addition, because there is published data on adult levels in this same community, our data add to our understanding of community-wide exposure to dioxins. Lastly, we agree that it is notable that PCBs and PCDFs were the primary contributors to TEQs in this analysis (see Conclusions).

Comment 2: I am also concerned about the size of this study. I understand very well the analytical expense of measuring dioxins. However, if any effects study is to be done, it is clear, and even emphasized by the results of this investigation, that individual monitoring must be done. The boys in this study are not very heavily contaminated by TEQ compared to other
populations. There are only 2/30 boys who have ANY detectable 2,3,7,8-TCDD.

Response: We agree that a larger study is required for a health effects study. However, this study was not intended to assess health effects but, instead, to characterize exposure and risk factors in an age group for which there is very little data and in a community in which excess exposure was hypothesized. This is more clearly stated in the manuscript.

In addition, this examination of dioxin levels in 30 boys was performed to generate preliminary data to guide the design of the larger study and to test study instruments and questionnaires. Based on experiences in this pilot study, the questionnaires have been revised and improved and our chemical analysis approach revised (larger sample volumes, better detection limits).

We are currently assembling a cohort of 550 eight- to nine-year old boys to follow them prospectively for growth and development. We have recruited over 450 boys to date. The low detection for TCDD in the study of 30 boys reported in this manuscript is likely partially due to the low volume of blood we collected. In the prospective cohort study, we are collecting 45 ml whole blood per child and anticipate higher detection rates.

Despite detection limitations, with few exceptions, the boys in this study had higher TEQ levels than have been reported in other non-occupationally exposed populations, including substantially older adults in whom higher exposure is expected based on age-related increases.

Comment 3: Also, most of the research on the effects of dioxins like chemicals demonstrates that prenatal exposure is the major determinant of adverse responses. There is no way to look at teenager and know what their early life exposure was. It would be more productive to examine their mothers. Given that this is a population which appears to have a high incidence of cryptorchidism, hypospadias, etc., effects which are believed to be associated with high prenatal estrogen exposure (NOT related to dioxins), there are clearly opportunities to ask questions about exposures early in life; but I do not think it likely that measuring teenager levels will be informative in relation to the effects in question.

Response: We agree with the reviewer’s comments and recognize the need to clarify the purpose and aims of the analysis of the 30 boys from the pilot study (see above response). The pilot study analysis presented in this manuscript was not designed to explore the relationship between cryptorchidism and hypospadias and dioxin-like compounds. We have clarified this in the manuscript. We have simplified the methods section to reflect these changes.

However, even in our pilot study, we recognized the importance of early life exposures, and consequently collected information on and evaluated maternal and pre-natal exposures and related confounders which could contribute to the boys’ early life exposure. For example, we collected breastfeeding duration of the index boy, prior breastfeeding duration of siblings, gestational age of subject, use of alcohol and smoking during pregnancy, gravidity, parity, and birth weight of subject. All of these factors related to maternal and early life history may contribute or modify early life exposures.

In our current ongoing prospective cohort study, we are collecting the mother’s blood and a detailed reproductive history (number of children and dates of birth, weeks of breast feeding the index son and other children, etc).

Comment 4: Response to the specific questions from Biomedcentral:
1. This is "a paper whose findings are important to those with closely related research interests.:}
2. The quality of the written English is acceptable.

Specific comments follow.

ABSTRACT:
Comment 5: The TEQ due to PCDDs is extremely low in this population demonstrating that whatever is causing the boys' exposure is not due to contaminated herbicides, chlorine bleaching of paper and pulp products, or incineration of general wastes. The predominance of PCDFs and PCBs contributing to the total TEQ suggests a PCB contamination and/or pentachlorophenol.

Response: We agree that the PCDDs are relatively low in this sample of 30 boys and that PCDFs and PCBs make major contributions to the total TEQ (see Conclusions).

BACKGROUND:
Comment 6: The usual t1/2 cited for 2,3,7,8-TCDD is 7 years. In fact, we now know that the half-life depends upon the body composition: the more fat, the longer the t1/2. Therefore, it is likely that for young boys the half-life will be less than 7 years, and clearly not 9 years.

Response: Although seven years is generally cited as the average TCDD half life in adults, and children likely have a shorter half life, it is clear that there is tremendous inter-individual variability in human TCDD metabolism and factors such as gender, age, body composition, and exposure level or exposure rate all likely play important roles. Recently updated toxicokinetic models suggest the TCDD t1/2 may range from 3 to 10 years in adults; there probably are not comparable models for children. We have revised our manuscript to reflect this potential variability.

METHODS:
Comment 7: 1. p.8, middle paragraph - Is the clinical chemistry method for determining serum lipids valid for this age group?

Response: This method is valid for this age group. The same method is also used by CDC for cord blood samples.

Comment 8: Using p>0.15 is unusual. It really does demonstrate that the sample size used in this study was not adequate for making conclusions.

Response: We agree that the sample size is small and therefore the study should be considered hypothesis generating rather than hypothesis testing. However, the wording of this section was somewhat unclear and we have rewritten to clarify. In fact, the p-value cutoff of 0.15 was specified to select potential predictors for inclusion in the multivariate models. This is a standard approach for model building (even when considering larger datasets). For example, Collett recommends first conducting univariate tests of association and then choosing variables to consider in a multivariate model, and states “when using this selection procedure, rigid application of a particular significance level should be avoided. In order to guide decisions on whether to include or omit a term, the significance level should not be too small; a level of around 10% is recommended.” (Collett, Strategy for Model Selection, in “Modelling Survival Data in Medical Research”. London: Chapman & Hall).

RESULTS:
Comment 9, p.13, top - How was the total WHO-TEQ for dioxins, furans and PCBs 40.9:
summing up the medians for the individual group of analytes results in a lower value.

Response: For means, it is the case that the mean of a sum is equal to the sum of the means (assuming there are no missing measurements for any subject). However, this relationship does not hold for medians unless the measurements have the same ordering among subjects for each exposure. The example below illustrates this fact:

Example 1: subject | PCDD teq | PCDF teq | SUM(PCDD+PCDF) teqs
---|---|---|---
1 | 1 | 60 | 61
2 | 2 | 14 | 16
3 | 8 | 46 | 54
4 | 21 | 8 | 29
5 | 68 | 2 | 70

mean = 100/5 = 20 mean = 130/5 = 26 mean = 230/5 = 46
median = 8 median = 14 median = 54

In the above example, the mean of the sum is equal to the sum of the means, but the sum of the medians is 22, which is not even close to the median of the sum. In practice, the more highly correlated two measures are, the closer the sum of medians will be to the median of the sum.

Comment 10. p. 13, 3rd paragraph - WHO recommends including the mono-ortho PCBs in the determination of the total TEQ. However, the contribution of mono-ortho PCBs to the total TEQ is unusually high. This would support my contention that the dioxin-like contamination suggested in this population is likely due to PCBs.

Response: We agree that mono-ortho PCBs made an important contribution to total TEQs, however, it is not unusually high. For example, when we compare percent contribution of mono-ortho PCBs to mean total TEQ values in our group with Seveso women (Warner et al, 2004) they are similar, at 24% in Chapaevsk and 22% in Seveso.

Comment 11. p.14, end of 1st paragraph - The high contribution of PCB 153 to the total PCB mass is unusual. Usually, this PCB accounts for approximately 20% of the total PCB mass. This might suggest what PCBs were involved in the contamination present in this area of Russia.

Response: PCB 153 is generally the most prevalent PCB congener in human serum. That said, its percent of the total varies somewhat and, among studies in which congener specific results are presented, obviously depends on the number of congeners assessed in the total. For the sum of congeners 138 + 153 + 180 measured in longitudinal studies of prenatal exposure and child development, 153 constituted approximately 30-50% of the sum of mean congener values (Korrick et al., J Expo Anal Environ Epidemiol, 2000) and similar proportions can be inferred from studies of adults (Gladen et al., Am J Ind Med, 1999).

Comment 12. p.14, bottom - The volumes of blood sampled were too small. Why were such small samples taken?

Response: Small samples were taken because our initial intent was to collect samples from all 246 boys and pool across sub-sets of boys based on demographics and medical histories. However, we believed this would compromise our ability to collect valuable data at the individual level. We therefore analyzed a subset of the individual samples, but we recognized that the volume was small. In the prospective cohort study we are collecting 45 ml of whole blood per boy.
Comment 13. p.15, bottom - The fact that there is a suggestion of a decrease in blood levels with increasing distance from the Khimprom factory is suggestive, but not persuasive from this data. Its really too bad that the design of this study did not sample enough boys.

Response: We agree that it is suggestive and in the larger study we will have sufficient power to explore this further.

DISCUSSION:
Comment 14. p.17, bottom - There is no reason to suspect that cryptorchidism or hypospadias are associated with dioxin. These effects have not been seen in experimental animals or wildlife, nor have they been seen in the several cohorts which have been studied. Delay in puberty has been seen in several animal studies.

Response: Our larger prospective cohort study is designed to explore the association between dioxin and dioxin-like compounds and puberty and growth. The interest in cryptorchidism and hypospadias grew out of our Russian collaborators initial interest in exploring potential exposures that may account for an increased risk of these developmental outcomes in the boys in Chapaevsk. In humans, hypospadias reflects in utero androgen insufficiency or inadequate action. In animal models, dioxins have been shown to suppress androgen production so it would be reasonable to extrapolate that hypospadias might be a consequence of in utero exposure to dioxin. We recognize that our study design was not optimal to explore this since we would need to measure or estimate levels of in utero exposure. To this end, we collected and archived samples from the mother of the boys in our prospective cohort study.

Comment 15. I do not agree that the levels in this population appear unusually high. It is key that the year of sampling be similar and that the age of the boys be the same.

Response: This study setting does not represent community exposures from an extreme accident (as, e.g., Seveso, Italy) but rather chronic moderate environmental contamination. Where the age of the boys are similar, the TEQs in this population were approximately twofold higher than has been reported elsewhere among community exposed individuals. The only non-occupationally exposed groups for which comparable or slightly higher levels have been reported (excepting Seveso) are in adults for whom higher levels are expected based on age alone. Thus, the levels in this sample appear higher than expected for the age group studied. Because the volume of serum for analysis was limited and the number of subjects was small, a definitive comparison to other populations awaits analyses in our larger study.

Comment 16. p. 19, end of 1st paragraph - The levels in the adult women in Shelekhova may not be very low given IF these women have lactated.

Response: We agree that this is a possibility and may account for low levels among these women.

CONCLUSIONS:
Comment 17. p.22, 1st paragraph - The mean WHO-TEQ of 44-45 year old adults of 61.2 ppt lipid is higher than one would expect for populations in the US or Western Europe today. Whether this is due to ongoing exposure or the product of past contamination is unknown.

Response: We agree with this observation. Based on these results, we expected boys in the same community (Chapaevsk) to have similarly elevated levels.
Comment 18.  p.23, end - While it is clearly feasible to measure dioxins in the blood of children, this study used too small a sample to make conclusions about whether the concentrations were unusually high or if there was any association between the children's current level and their health status. Knowing this group of researchers, it is hard for me to believe that they did not do the appropriate power calculations before they started their study. It is time to move past the claim that it is too expensive to measure dioxins and therefore they only examine a limited number of subjects. Either do the study correctly, or don't do it at all!

Response:
We agree that a larger study is required for a health effects study. Our original pilot study included 246 boys, of whom 221 provided blood samples. Analyzing dioxin samples on this large a group would have provided sufficient power to detect differences and associations of scientific interest, and this was the size of the study by design to allow sufficient power. However, the current study was not intended to assess health effects but, instead, to characterize exposure and risk factors in an age group for which there is very little data and in a community in which excess exposure was hypothesized.

In addition, this examination of dioxin levels in 30 boys was performed to generate preliminary data to guide the design of the larger study and to test study instruments and questionnaires. Based on experiences in this pilot study, the questionnaires have been revised and improved and our chemical analysis approach revised (larger sample volumes, better detection limits). Pilot studies are essential to optimizing the design of large studies. For example, we recognized after testing this first group of 30 subjects that our sample volume was too low. We also worked with CDC to refine the approach to dioxin assays, so that improved detection of low exposures could be achieved for application to our planned larger studies. However, we believe the exposure information obtained in this study provides valuable insight into the relative magnitude and exposure risk factors likely characteristic of the study population. We agree that larger studies are essential. In the revised manuscript we explicitly state and clarify the aims of this pilot study and how it relates to our planned larger study.

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Response: We agree and have revised this accordingly. This was the aim of our larger ongoing study.

Comment 2-pg 3 para 2 ln 3 please provide information about what is known about childhood and adolescent exposure and effects rather this statement

Response: We have revised as suggested. Please see page 4-5 in revised manuscript. In particular, we have now included additional background studies conducted by Den Hond et al. (2002), Gladen et al. (2000), and Guo et al. (1993).

Comment 3-provide some information on environmental levels
Response: As suggested, we have briefly included data on environmental levels in soil and drinking water collected by our co-investigator (Dr. Revich). Please see page 6.

Comment 4-dioxin-like compounds would be preferable than to refer to these chemicals as "dioxins"

Response: We agree and have revised accordingly throughout the manuscript.

Methods
Comment 5- The sentence beginning "Of the 246 boys" is awkwardly written

Response: We have simplified and revised this paragraph on page 6.

Comment 6- It is not clear what is meant by "number of targeted control boys was proportionate to the average of the percentage of cases." Proportionate to what average of the percentage of what?

Response: This was referring to sampling in our larger pilot study and since it was not directly relevant to the 30 boys sampled here we have deleted it from the text. We feel this clarifies this section.

Comment 7-Other than splitting the 30 samples by case and control status, how else where these samples selected for analysis?-was it intentionally split?

Response: Yes, samples were intentionally split by case-control status, but were otherwise a convenience sample that was not related to any factors that could be related to the actual exposure levels. In selecting the 30 samples, we were blinded to any information apart from case-control status.

Comment 8-How many cc's of blood were taken? Which PCBs were measured?

Response: We obtained approximately 12 cc of blood, thus approximately 6 cc of serum. We realize this is a low volume. Please see response to reviewer 1 comment #4. The measured congeners are listed in Tables 2 and 3 and those tables are now referenced in the methods.

Comment 9- What was the LOD for TCDD? So you did not divide those samples below the detection limit by squared root of 2? Which lipids were measured?

Response: The method detection limit for 2,3,7,8 TCDD in 10 g sample is 2.1 pg/g lipid, however, since all samples had different weights, different percent lipids and percent recoveries, the DLs for TCDD in our study samples ranged from 1.5 to 16.5 pg/g lipid.

We have used uncensored data and retained measurable values below the DL in our dataset, as described in Korrick et al, 2000 and also in our paper (p. 9, last paragraph).

The four lipid groups (free or non-esterified cholesterol, cholesterol esters, triglycerides and phospholipids) were measured to calculate total lipids using “enzymatic method” (Akins et al., 1989).

Comment 10-Why were PCBs not measured on all (pg 10, In9)
Response: Results for some samples could not be reported because one or more of the analytical QA/QC criteria were not met. Therefore, the denominator may be less than 30. This is footnoted in Tables 2 and 3.

Results

Comment 11-Table 1-it is not necessary to present the median, min and max; also best not to use M+SD as the column heading when there are n, % in some lines-it is confusing

Response: Since the mean and median are similar we have deleted the median and min and max as suggested. We have also restructured the column headings for improved clarity.

Comment 12-Table 2-most of the data are 0’s with few above the DL. Therefore, perhaps this information can be summarized more succinctly, e.g. median and IQR or GM and SD-too much space for amount of data. However, it would be helpful to know the detection limits.

Response: We agree and have only included the median and 25th and 75th percentiles. As suggested we have added the average DLs.

Comment 13-Fig 2 is not legible at all and Fig 1 is of poor quality (faxed?)

Response: All figures were sent as electronic files. The poor legibility may have occurred as a result of the transmission of the files or the use of a different program to open them. We have resent all figures as pdf files with the resubmission.

Comment 14-Table 5 is presented after Table 6 for the first time. It is not necessary to include both.

Response: This was an error. In the paragraph describing Table 5 we meant to refer to table 4 (not table 6). This has been corrected.

Comment 15-Table 6 is log base 10 or natural log? It would be more comprehensible if it were base 10. How was it determined that the mean sum increased by 30%. The description of the multivariate tables is not clear.

Response: We used log base 10 (now explicitly noted). Because the outcome variable is the log of dioxins, when you back transform the regression coefficient, the change in dioxins per unit change in the predictor is a multiplicative factor (a proportion) rather than an additive factor. We have revised the description of the multivariate table.

Comment 16-What is the rationale for putting PCB 118 in the model?

Response: In exploring the best model that predicted dioxin-like compounds, we found that PCB118 was the strongest predictor and may therefore be of importance in our future work estimating dioxin exposure for several hundred individuals. In addition, PCB118 is by far the most prevalent dioxin-like PCB (mono-ortho substituted) and therefore is often considered in analyses of dioxin-like health effects. Models with other PCB congeners were run and PCB 118 was found to be a stronger predictor.