Exposure to lead and vaccine-specific IgG titers in South African children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE): A longitudinal study

Thomas Z. Di Lenardo,[1] Brian J. Ward,[2,3] Stéphane Pillet,[2] Koren Mann,[3,4] Riana Bornman,[5] Muvhulawa Obida,[5] Jonathan Chevrier[1,*]

[1] Department of Epidemiology, Biostatistics and Occupational Health, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

[2] Research Institute of the McGill University Health Centre, Montreal, Quebec, Canada

[3] Division of Experimental Medicine, Department of Medicine, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

[4] Gerald Bronfman Department of Oncology, Faculty of Medicine, McGill University, Montreal, Quebec, Canada

[5] University of Pretoria Institute for Sustainable Malaria Control and School of Health Systems and Public Health, University of Pretoria, Pretoria, South Africa

*Address Correspondence to: Jonathan Chevrier, Department of Epidemiology, Biostatistics and Occupational Health, Faculty of Medicine, McGill University, 1020 Pine Avenue West, Montreal, QC, Canada, H3A 1A2; Telephone: 514 398-8598; Fax: 514 398-4503; Email: jonathan.chevrier@mcgill.ca

Highlights

- Exposure to lead was inversely associated with tetanus antibody titers overall.
- Lead and Hib titers were inversely associated among children of HIV positive mothers.
- Lead was inversely associated with measles antibody titers in girls but not in boys.
- Associations were found in a population with low exposure to lead.

Abbreviations:

3-PBA: 3-phenoxybenzoic acid

4-F-3-PBA: 4-fluoro-3-phenoxybenzoic acid

ARV: Antiretroviral

ASC: Antibody-secreting cells

BLL: Blood lead level

cis-DBCA: *cis*-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane carboxylic acid

cis-DCCA: *cis*-3-(2,2,-dicholorvinyl)-2,2-dimethyl-cyclopropane carboxylic acid

DDT: Dichlorodiphenyl trichloroethylene

DDE: Dichlorodiphenyl dichloroethane

DTaP-IPV: Diphtheria, tetanus, pertussis - inactivated polio vaccine

Hib: Haemophilus Influenzae type B

HIV: Human immunodeficiency virus

IgG: Immunoglobulin G

LOD: Limit of detection

LOQ: Limit of quantification

Pb: Lead

SRBC: Sheep red blood cells

trans-DCCA: *trans*-3-(2,2,-dicholorvinyl)-2,2-dimethyl-cyclopropane carboxylic acid

VHEMBE: Venda Health Examination of Mothers, Babies and their Environment

Abstract

Background: While successes have been achieved in reducing global exposure to lead, few studies have investigated the potential health effects of low-level exposure (e.g. blood lead levels [BLLs] below the CDC reference level of 5 µg/dL), particularly among children from low- and middle-income countries. In addition, lead is immunotoxic in animals but human data on immune response to vaccines is limited. Our aim was to determine whether low-level exposure to lead is associated with humoral response to vaccines among rural South African children.

Methods: We used data from the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), a birth cohort study conducted in Limpopo, South Africa. BLLs were measured in whole blood collected at age 1 and IgG titers for measles, tetanus and *Haemophilus influenzae* type B (Hib) were determined at age 3.5 years among 425 fully-vaccinated children.

Results: BLLs were low (median=1.90 µg/dL) and 94% of children had a BLL below 5 µg/dL. Overall, BLLs were associated with higher risks of having IgG titers below the protective limit for tetanus (RR=1.88 per 10-fold increase; 95%CI=1.08, 3.24) but not measles (RR=1.02; 95%CI=0.26, 3.95) or Hib (RR=0.96; 95%CI=0.54, 1.71). BLLs were also associated with low Hib IgG titers among children exposed to HIV *in utero* and with low measles IgG titers among females. In contrast, the association with measles IgG titers was positive among males.

Conclusion: Low-level exposure to lead may compromise the humoral response to vaccines. Children exposed to HIV *in utero* and females may be particularly susceptible.

Keywords: Lead, Vaccine response, Measles, Tetanus, Haemophilus influenzae type B, Immunotoxicity, Early childhood vaccines

1. Introduction

Despite major successes in reducing environmental exposure to lead in many areas of the world, this metal continues to contribute to the global burden of disease, particularly among children from low- and middle-income countries (1, 2). While the neurological effects of lead have historically been the primary focus of attention, lead affects almost every system in the body including the haematopoietic, cardiovascular, reproductive, gastrointestinal, and renal systems (3, 4). Animals studies also show that lead is an immunotoxicant. For example, mice exposed to lead acetate in drinking water for 10 weeks produced fewer splenic antibody-secreting cells (ASC) after immunization with sheep red blood cells (SRBC)(5, 6). In a similar study, male mice immunized with SRBC had lower antigen-specific antibody titers and fewer ASC after exposure to tetraethyl lead for 3-weeks (7). In humans, numerous studies suggest that exposure to lead is associated with changes in total immunoglobulin and serum cytokine concentrations as well as circulating lymphocyte subpopulations (8-21) but results have been inconsistent and such non-specific markers provide limited information about the potential impact of lead on functional immunity.

Because the generation of antigen-specific antibodies requires the appropriate interaction of multiple immune cells and pathways, measuring vaccine-induced antibody titres following immunization represents a more comprehensive evaluation of functional immunity in which both antigen dose and the developmental timing of inoculation are standardized. To our knowledge, such studies have only been conducted in two populations. Among children aged 9 months to 6 years recruited through the Women, Infants and Children (WIC) program in Missouri, USA, (n= 193) blood lead levels (BLLs) (mean=10.4 µg/dL, range=1 to 50) were associated with decreased

vaccine-induced antibody titers for diphtheria and rubella, but not tetanus (22). More recent studies of children aged 3-7 years living in an electronic waste recycling area in Guangdong province, China (n= 284) found that BLLs (median=9.4 µg/dL) were associated with lower IgG antibody titers against pertussis, diphtheria, polio, measles (23) and hepatitis B (23, 24). However, these children were exposed to high levels of multiple other contaminants that could affect immune function thus confounding possible associations with lead. In addition, all of these studies were conducted in populations with moderate to high exposure. While BLLs below 10 µg/dL are associated with poorer neurodevelopment in children (25, 26), few studies have investigated the health effects of BLLs below 5 µg/dL, the U.S. Centers for Disease Control and Prevention (CDC) reference level (27, 28).

To our knowledge, no prior study has examined the relation between lead exposure and vaccine-induced immunity in a population with low exposure to lead. African children may be particularly susceptible to the immunotoxic effects of environmental contaminants due to poverty, malnutrition and HIV. We thus used data from an established birth cohort study to investigate whether exposure to lead was associated with vaccine-induced antibody response among children from a rural area of Limpopo Province, South Africa who experience low-level exposure to this heavy metal.

2. Methods

2.1. Study Participants

We used data from the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), a prospective birth cohort study investigating the environmental determinants of

health and development among children residing in the rural Vhembe district of Limpopo Province, South Africa. Mothers were recruited when they presented for delivery at Tshilidzini Hospital in the town of Thohoyandou between August 2012 and December 2013. Women were eligible if they were at least 18 years of age, spoke Tshivenda (the most commonly spoken language in the area) at home, had contractions at least 5 minutes apart, were free from malaria during the index pregnancy, lived within 20 km of the hospital and planned to remain in the area for at least 2 years, and delivered a live singleton. Of 920 eligible mothers, 152 refused participation, 3 did not provide a blood sample (a requirement for participation in the VHEMBE study) and 13 did not complete the baseline questionnaire at delivery, leaving 752 women enrolled. A home visit was also completed one week postpartum with 723 mothers. Children were subsequently followed when they were aged 1, 2 and 3.5 years in our field office at Tshilidzini Hospital. A total of 700 children were assessed at age 1 year, 501 of whom provided sufficient blood volume for blood lead measurements. Of these, 482 completed the 2-year visit and 459 completed the 3.5-year visit and provided sufficient blood volume for vaccine-specific serum antibody measurements. We excluded 37 children who did not receive the two scheduled measles immunizations, and 71 children who did not received all four scheduled DTaP-IPV/Hib immunizations by the 3.5-year visit, leaving final sample sizes ranging between 388 (tetanus and *Haemophilus Influenzae* type b [Hib]) and 422 (measles) for analyses investigating vaccine protection. Children who contracted measles before the 3.5-year visit (n=16) were excluded from analyses in which antibody titers were expressed continuously, leaving a sample size of 406 for these analyses. Informed consent was obtained from all women or caregivers prior to participation. This study was approved by the ethics committees of McGill University, the

University of Pretoria, the Limpopo Department of Health and Social Development and Tshilidzini Hospital.

2.2. Data Collection

Questionnaire-based interviews were conducted with the mother or a primary caregiver by bilingual staff (Tshivenda and English) in the local Tshivenda language at each study visit. At delivery, questionnaires were administered to mothers prior to hospital discharge to collect information on maternal sociodemographics, health and pregnancy history, including the date of the last menstrual period. Maternal age, marital status, parity, and family income were recorded. Poverty was defined as an income below R386 per person per month based on Statistics South Africa guidelines (29). Because income may not fully represent socioeconomic status in the study area, we also constructed a wealth index via principal component analysis based on Demographic and Health Surveys methodology (30) using information from the delivery questionnaire and the 1-week home visit (31). Breastfeeding duration was determined based on data from the 1, 2 and 3.5-year visit questionnaires and measles diagnosis was based on maternal or caregiver report at the 3.5-year visit. Immunization dates for the routine measles (Rouvax, Sanofi-Pasteur) and DTaP-IPV/Hib (Pentaxim, Sanofi-Pasteur) vaccines were abstracted by registered nurses from the child's *Road to Health* booklet, which is kept by caregivers and serves as a medical record. Hospital staff measured birth weight using a Tanita BD-815U neonatal scale (Tanita Corporation of America, Inc., Arlington Heights, Il, USA). Data on birth weight, gestational age at birth, and maternal or child anti-retroviral drug prescriptions were abstracted by two registered nurses from maternal and child medical records. Maternal HIV status was ascertained from self-reported diagnosis or anti-retroviral drug prescription. Gestational age was

determined based on date of last menstrual period from questionnaire and medical record data as described by Chevrier *et al.* (32).

Malnutrition is common in the VHEMBE population and may confound and/or modify associations between lead and immune function. We assessed chronic child malnutrition based on stunting at age 1 year, which was defined as length-for-age below two standard deviations from the mean according to WHO standards (33). Recumbent length was measured in triplicate using a Seca 417 measuring board (Chino, CA, USA) and averaged, following U.S. National Health and Nutrition Examination Survey (NHANES) protocols (34).

2.3. Insecticide measurements

The VHEMBE population resides in an area where Indoor Residual Spraying with dichlorodiphenyl trichloroethylene (DDT) and pyrethroids is conducted annually to control malaria, which may lead to elevated exposure to insecticides (35-37). Because these insecticides may impact immune function, we considered them as potential confounders. Lipid-corrected DDT and dichlorodiphenyl dichloroethane (DDE), DDT's breakdown product, were measured in maternal serum collected at enrollment by the Emory University Environmental Health Laboratory (Atlanta, GA, USA) via high-resolution gas chromatography–isotope dilution mass spectrometry (GC-MS) (38). The Institut National de Santé Publique du Québec measured the concentration of 5 pyrethroid metabolites in maternal urine collected at enrollment using GC-MS (39): 4-fluoro-3-phenoxybenzoic acid (4-F-3-PBA), cis-3-(2,2-dibromovinyl)-2,2-dimethyl-cyclopropane carboxylic acid (cis-DBCA), cis-3-(2,2,-dicholorvinyl)-2,2-dimethyl-cyclopropane carboxylic acid (cis-DCCA), trans-3-(2,2,-dicholorvinyl)-2,2-dimethyl-cyclopropane carboxylic

acid (trans-DCCA), and 3-phenoxybenzoic acid (3-PBA). Concentrations were corrected for urine dilution via specific gravity, which was determined using an Atago PAL-10S refractometer (Bellevue, WA, USA). Insecticide concentrations were $\log_{10}$-transformed for analysis.

2.4. Blood lead measurement

Lead analysis was conducted by Lancet Laboratories (Johannesburg, South Africa), a member of the Royal College of Pathologists of Australasia Quality Assurance – Trace Element Programme. Lead concentrations were measured in triplicate in whole blood samples collected at the 1-year visit on an Agilent 7900 Inductively Coupled Plasma-Mass Spectrometer with an Octopole Reaction System. Contamination-free vessels and procedures were used throughout, and validation of results was accomplished by including certified standards and quality control samples. Seronorm Trace Elements (Sero LTD., Billingstad, Norway) and Clinchek Controls (RECIPE, Munich, Germany) were used for this purpose. The limit of detection was 0.1 μg/dL.

2.5. Vaccine-specific IgG antibody measurement

We measured serum IgG specific to vaccines representing three distinct classes, namely tetanus (aluminum-salt adjuvanted protein), measles (live-attenuated virus) and Hib (conjugated polysaccharide-subunit) by enzyme-linked immunosorbent assay (ELISA) at the Research Institute of the McGill University Health Centre. Briefly, serum collected at the 3.5-year time point was diluted 1:100 in sample buffer as per the manufacturers' protocols and deposited on 96-well plates precoated with either tetanus toxoid (GWB-FCBEAB; Genway Biotech Inc., San Diego, CA), measles antigen (GWB-984A72; Genway Biotech Inc., San Diego, CA) or Hib polyribosylribitolphosphate (RE56351; IBL International, Hamburg, Germany; fulfilled by

Affinity Diagnostics, North York, ON) for one hour. Antigen-specific IgG was detected using a secondary horseradish-peroxidase conjugated anti-human IgG antibody. After 3,3′,5,5′-tetramethylbenzidine substrate reaction, absorbance was measured at 450 nm using an EL800 microplate reader (BioTek Instruments Inc., Winooski, VT). Duplicates were run for 10% of the samples for quality control purposes. Antibody concentrations were interpolated from standard curves created using serially-diluted international standards for each vaccine antigen plated in duplicate. Standards for tetanus (TE-3), measles (97/648) and Hib (12/306) were purchased from the National Institute for Biological Standards and Control (NIBSC; Blanche Lane, Ridge, Herts, UK) were serially diluted 1:5 and included on each 96-well plate, in duplicate. The limit of detection for tetanus, measles and Hib ELISAs were 0.05 IU/mL, 0.05 IU/mL and 0.06 μg/mL, respectively, based on the lowest standard containing analyte and blank as previously described (40). Protective cut-offs were based on previously-published values of 0.1 IU/mL for tetanus (41-43), 250 mIU/mL for measles (44, 45) and 1 μg/mL for Hib (41, 46).

2.6. Statistical Analysis

We used Pearson's correlations and analysis of variance to evaluate bivariate associations. We used multivariable Poisson regression with a robust (Huber-White) variance estimator or multivariable linear regression to evaluate associations between lead and IgG titers expressed categorically (below vs above protective cut-offs) or continuously, respectively. Lead concentrations were $\log_{10}$-transformed to reduce the influence of outliers and IgG titers below the LOQ were assigned machine-read values. In linear regression models, antibody titers were $\log_{10}$-transformed to normalize residuals and beta coefficients were transformed using $(10^{\beta} - 1)*100$. Reported results thus represent a relative risk of non-protection (Poisson models) or

percent change in antibody titers (linear models) associated with a 10-fold increase in BLLs. LOWESS plots suggested that the linearity assumption was not violated for associations between (log_{10}-transformed) BLLs and (log_{10}-transformed) tetanus or measles antibody titers (Supplemental Figure S1). Plots provided some evidence of a U-shaped curve for the association between lead and Hib, but evidence for departure from linearity was limited as the addition of a quadratic term did not significantly improve model fit based on an F-test. Lead was thus expressed linearly for all models. We considered the following variables as potential confounders: maternal age (continuous), education (<12th grade, 12th grade, >12th grade), marital status (married or living as married, not married), serum total polychlorinated biphenyls, *p,p'*-DDT and *p,p'*-DDE concentrations (continuous), and urinary pyrethroid metabolite concentrations (continuous); alcohol consumption (any, none), smoking (ever, never), exposure to environmental tobacco smoke (ever, never), and maternal HIV status (positive, negative) during pregnancy; household income per capita (continuous) and wealth index (continuous); and child sex, birth weight (continuous), gestational age at birth (continuous), delivery method (cesarean, vaginal), duration of breast feeding (in months, continuous), stunting at age 1 year (height-for-age <2 SD, ≥2 SD) and time since last measles or DTap-IPV/Hib vaccine at the time of sample collection for IgG titer measurement (in days, continuous). Variables were included in models if they were at least moderately associated in bivariate analyses with BLLs and one of the IgG titers based on p-values <0.20. Final models included maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine. Because IgG titers wane over time, time since last vaccine may be an important confounder. We thus applied a 3-knot restricted cubic spline to this variable to limit the potential for residual confounding. Missing covariate values (< 1.9%) were imputed at random based on observed probability

distributions. We also investigated effect modification by child sex, *in utero* exposure to HIV (based on maternal status during pregnancy) and child malnutrition (based on stunting at age 1 year) by including cross-product terms in models. All models were corrected for potential selection bias by applying inverse probability-of-censoring weights. Probability of censoring was determined via multivariable logistic regression. Weights varied between 1.3 and 3.2, suggesting no practical violation of the positivity assumption.

We ran sensitivity analyses to evaluate the robustness of our results. In order to evaluate whether the cut-off selected for protection influenced results, we re-ran all Poisson models using higher and lower cut-off values. Higher values were determined based on titers for which a booster vaccine is generally recommended and lower values were selected as the mid-point between original cut-off values and the LOD. In addition, because categorizing continuous independent variables may introduce bias (47, 48), we ran quantile regressions predicting the 33^{rd} (approximately equal to values for protection for tetanus and Hib) and 25^{th} percentiles. We also re-ran linear regression models excluding outliers as identified using the generalized extreme studentized deviate many-outlier procedure (49). Finally, we re-ran all regressions including only children with BLLs below 5 µg/dL. All analyses were conducted using STATA version 13 (StataCorp, College Station, TX).

Table 1. Demographic characteristics of VHEMBE study participants (n=425).

Maternal characteristics		
Maternal age (years), mean ± standard deviation	26.8	± 6.4
Education, n (%)		
< 12th grade	238	(56.0)
Grade 12	125	(29.4)
> High school	62	(14.5)
Parity, n (%)		
0	175	(41.2)
1	115	(27.0)
2+	135	(31.8)
Alcohol consumption, n (%)		
Yes	38	(8.9)
No	387	(91.1)
Smoking, n (%)		
Yes	4	(0.9)
No	421	(99.1)
Environmental tobacco smoke exposure, n (%)		
Yes	157	(37.0)
No	267	(63.0)
HIV status, n (%)		
Positive	54	(12.7)
Negative	371	(87.3)
Child characteristics		
Child age (months), mean ± standard deviation	42.9	± 1.20
Sex, n (%)		
Boy	218	(51.3)
Girl	207	(48.7)
Preterm (<37 weeks), n (%)		
Yes	55	(12.9)
No	370	(87.0)
Low birth weight (<2500g), n (%)		
Yes	31	(7.3)
No	394	(92.7)
Delivery method		
Vaginal birth	322	(75.8)
Cesarean	103	(24.2)
Duration of breastfeeding		
≤ 18 months	244	(57.4)
> 18 months	181	(42.6)
Stunting at age 1, n (%)		

Yes	68	(16.0)
No	356	(83.7)
Household characteristics		
Below food poverty level (R386/month per capita)		
Yes	247	(58.1)
No	178	(41.9)

Totals may not add to 425 due to missing data.
Percentages may not add to 100% due to rounding

3. Results

3.1. Population characteristics

Table 1 shows participant characteristics. All participants were black/of African descent. Mean maternal age at delivery was 26.8 years (standard deviation [SD]=6.4) and most women had a low level of education (56% did not complete high school) and low income with 58% being below the South African poverty level. About 13% of women were HIV positive and few smoked (1%) or drank any alcohol (9%) during pregnancy. The majority of mothers (59%) were parous prior to the birth of the index child. About half (49%) of the children were girls, 13% were born preterm (<37 weeks) and 7% had a low birth weight (< 2500 g). None of the HIV-exposed children became HIV-positive based on the child's *Road to Health* record. A large proportion (43%) of children were breastfed beyond 18 months and 16% of children were stunted at 1 year of age. The mean age of children at the 3.5-year visit was 42.9 months (SD=1.2).

3.2. Blood lead and serum antibody concentrations.

All blood lead concentrations were above the limit of detection (Table 2). Concentrations were generally low with a median of 1.9 µg/dL (interquartile range (IQR)=1.3, 2.8); 26 (6%) children had a BLL above 5 µg/dL and 3 (<1%) children had a BLL above 10 µg/dL. Blood lead was

Table 2. Child lead serum concentrations (ug/dL) at 1 year and vaccine antigen-specific IgG titers at 3.5 years among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa.

						Percentile					
	n			GM[a]	± GSD[b]	Min	10	25	50	75	90	Max
Lead	425			1.94	± 1.20	0.1	1.0	1.3	1.9	2.8	4.1	11.0
		Above cut-off	Below cut-off									
Measles	422	382	40	1.82	± 1.67	<LOQ	0.47	1.33	2.40	3.27	4.19	7.94
Tetanus	388	268	120	0.22	± 0.25	<LOQ	0.06	0.09	0.16	0.47	1.27	6.09
Hib	388	269	119	1.99	± 2.69	0.11	0.23	0.73	2.53	6.67	9.49	12.91

Limits of quantification LOQs: 0.06 IU/mL (Measles IgG); 0.06 IU/mL (Tetanus IgG); 0.08 µg/mL (Hib IgG).
Antibody cut-off values: 0.25 IU/ml (Measles); 0.10 IU/ml (Tetanus); 1.0 µg/mL (Hib)
[a] Geometric mean
[b] Geometric standard deviation

associated with duration of breastfeeding and maternal HIV status. Median serum IgG concentrations for tetanus, measles and Hib were 0.16 IU/mL (IQR=0.09, 0.47), 2.40 IU/mL (IQR= 1.32, 3.25) and 2.53 μg/mL (IQR= 0.73, 6.67), respectively. The proportion of children with serum antibodies below the protective cut-off values were 30.9% (n = 120) for tetanus, 9.5% (n = 40) for measles and 30.6% (n = 119) for Hib. Antibody titers for the three vaccine targets were associated with different variables. Tetanus titers were inversely associated with maternal urinary *cis*-DBCA concentrations; measles IgG titers were inversely associated with maternal age; and Hib titers were inversely associated with maternal HIV status.

3.3. Associations between lead concentrations at age 1 year and vaccine antibody titers at age 3.5 years

BLLs were inversely related to tetanus antibody titers. A 10-fold increase in BLL was associated with a 28% decrease (95%CI= -52.16, 8.72) in anti-tetanus IgG and a doubling in the risk (Relative Risk [RR] = 1.88; 95% CI= 1.08, 3.24) of having tetanus IgG titers below the protective cut-off (Table 3). No overall association was observed between lead levels and either measles or Hib antibody titers, whether they were expressed continuously (β = 14.81%; 95% CI= -18.82, 62.36, and β = -15.53%; 95% CI= -52.15, 49.12, respectively) or categorized based on cutoffs (RR = 1.02; 95% CI= 0.26, 3.95, and RR 0.96; 95% CI= 0.54, 1.71, respectively). Findings were similar in sensitivity analyses when modifying antibody titer cut-offs within the equivocal range of protection or based on quantile regression (Supplemental Tables S1-S3).

Table 3. Associations between blood lead concentrations at 1 year and vaccine-specific IgG titers at 3.5 years among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa.

	n	β%	95% CI
Continuous			
Measles	406	14.81	(-18.82, 62.36)
Tetanus	388	-27.88	(-52.16, 8.72)
Hib	388	-15.53	(-52.15, 49.12)
	n	RR	95% CI
Dichotomous[a]			
Measles	422	1.02	(0.26, 3.95)
Tetanus	388	1.88*	(1.08, 3.24)
Hib	388	0.96	(0.54, 1.71)

*p<0.05

[a]Antibody cut-off values: 0.25 IU/ml (Measles); 0.10 IU/ml (Tetanus); 1.0 µg/mL (Hib)

Models adjusted for maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine

Table 4. Associations between blood lead at 1 year and IgG titers at 3.5 years by sex, maternal HIV status and stunting among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa.

		Measles	Tetanus	Hib
		β% (95% CI)	β% (95% CI)	β% (95% CI)
Child Sex	Male	69.82 (7.15, 163.0)†	-25.87 (-58.31, 25.89)	25.89 (-39.74, 169.15)
	Female	-25.87 (-52.14, 12.20)	-29.21 (-62.85, 34.90)	-47.52 (-74.88, 9.65)
Maternal HIV status	Negative	-8.80 (-33.93, 23.03)‡	-32.39 (-57.34, 4.71)	0.00 (-45.05, 81.97)†
	Positive	378.63 (123.87, 923.29)	25.89 (-41.11, 169.15)	-77.61 (-93.83, -20.56)
Stunting at age 1	No	-6.67 (-35.43, 31.83)†	-27.56 (-56.35, 17.49)	-29.21 (-61.09, 28.82)
	Yes	113.80 (9.65, 326.58)	-25.87 (-64.52, 51.35)	47.91 (-56.34, 389.78)
		Measles	Tetanus	Hib
		RR (95% CI)	RR (95% CI)	RR (95% CI)
Child Sex	Male	0.22 (0.06, 0.87)‡	1.77 (0.82, 3.82)	0.85 (0.40, 1.83)
	Female	5.80 (1.14, 29.48)	1.99 (0.93, 4.27)	1.14 (0.52, 2.49)
Maternal HIV status	Negative	2.76 (0.70, 10.97)‡	1.99 (1.11, 3.56)	0.76 (0.42, 1.38)†
	Positive	0.07 (0.02, 0.33)	1.07 (0.22, 5.13)	3.79 (0.96, 15.06)
Stunting at age 1	No	1.54 (0.37, 6.47)	1.67 (0.89, 3.13)	1.12 (0.59, 2.13)
	Yes	0.35 (0.36, 3.31)	3.21 (0.96, 10.70)	0.70 (0.27, 1.72)

*$p_{int}<0.1$; †$p_{int}<0.05$; ‡$p_{int}<0.01$

Models adjusted for maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine

We found limited evidence of effect modification by sex, maternal HIV status during pregnancy or child malnutrition for associations between lead and tetanus antibody titers (Table 4). However, maternal HIV status modified associations between lead and sub-protective Hib IgG titers ($p_{interaction}$ = 0.032) with associations being found among children of HIV positive mothers (RR = 3.79; 95% CI= 0.96, 15.06) but not among children of HIV negative mothers (RR = 0.76; 95% CI: 0.42, 1.38). This trend remained when Hib IgG titers were expressed continuously; a 10-fold increase in lead concentrations was associated with a 77% decrease in Hib titers (95% CI: -93.8, -20.6) among children of HIV positive women but the association was null in children of HIV negative mothers (β = 0.00; 95% CI: -45.0, 82.0).

We also found that the association between lead and measles IgG titers was modified by sex. Lead concentrations were inversely associated with measles antibodies among females, but associations were positive among males when antibody titers were categorized or expressed continuously. In addition, we observed inverse associations among children whose mothers were HIV negative during pregnancy and who were not stunted at age 1, however, estimates were imprecise possibly due to the low proportion of children with measles IgG titers below protection (Supplemental Tables S2 & S3).

Excluding outliers from linear regressions and restricting analysis to children with BLLs below 5 µg/dL did not materially affect the results described above (data not shown).

4. Discussion

We found that BLLs at age 1 were associated with an increase in the risk of tetanus antibody titers being sub-protective among South African children aged 3.5 years. Only two human studies have investigated associations between lead exposure and antibody response to tetanus, yielding inconsistent results. While a study conducted in Guangdong province, China, found that blood lead levels were inversely associated with tetanus antibodies (23), another study among Missouri children reported no association (22). However, the trend in the latter study was towards decreasing tetanus IgG titers and, given the moderate sample size (n=193), the null result may have been due to limited statistical power.

After investigating effect modification by sex, we found that lead concentrations were inversely associated with measles antibodies among girls but that the association was positive among boys. In human studies, exposure to lead has been associated with lower total IgG and increased total IgE and allergic disease (50, 51) in girls but not in boys. Experimental studies suggest that male and female rodents respond differently to lead exposure (52, 53). For instance, female offspring of F344 rats exposed to lead acetate in drinking water had decreased delayed-type hypersensitivity responses, monocyte and basophil counts, as well as increased neutrophil counts and splenic weights at 13 weeks of age (52). No changes were observed in the males for these same endpoints. On the other hand, male mice exposed to lead had significantly higher IL-12 and lower IL-10 production compared to controls, but no similar effect was observed in females (53). Taken together, these results suggest that lead exposure may increase the inflammatory response in male offspring and suppress it in females. This effect may occur through endocrine disruption since lead interferes with sex hormones (54-58), which in turn modulate immune response (59-61). It is not clear why we observed effect modification by sex for associations with measles

IgG, but not with tetanus or Hib. However, these vaccines are different in character and the measles vaccine, which is live, may more closely resemble an actual infection than the tetanus or Hib vaccine.

Additionally, we found that lead was associated with low Hib titers and a greater risk of titers being below the protective cut-off among children of HIV positive mothers but not in children of HIV negative mothers. It is possible that exposure to HIV or anti-retroviral therapy may increase susceptibility to the immunotoxic effects of lead since children infected with HIV through maternal transmission have been reported to have lower vaccine-specific IgG titers (62-65). However, in the present study, none of the HIV-exposed children were infected and it is unclear whether the immune response to vaccines is affected in such children as studies on this topic are limited by small sample sizes and yielded inconsistent results (63, 65, 66). Additional research on the mechanism by which HIV-exposed, uninfected children may be more susceptible to the immunotoxic effects of lead is warranted, including the possibility that antiretroviral drugs may potentiate the toxic effects of lead (67).

This study had several strengths. To our knowledge, this is the first study to investigate associations between lead and vaccine-specific antibody responses in a population with low exposure to lead. It is also the first study of the immunotoxic potential of lead in African children. Although residual confounding remains a possibility, we considered numerous variables as potential confounders, including environmental exposures, and had virtually complete data for all variables. In addition, unlike prior cross-sectional studies examining associations between lead exposure and immune outcomes, we measured blood lead at 1 year of

age and examined antibody titers prospectively at age 3.5 years. Because the antibody response to vaccines is typically detectable within 14 days following immunization, lead exposure at the time of vaccination is expected to be most relevant. Thus, measuring BLL at age 1 conferred an additional advantage in that this timepoint falls during the measles (at ages 6-9 and 12-18 months) and DTaP-IPV/Hib (at ages 6, 10, 14 weeks, and 18 months) immunization schedule. Since blood lead levels change throughout childhood (68-70), prior studies, which used measurements taken later in life, may have been affected by measurement error. Finally, we conducted multiple sensitivity analyses to evaluate the robustness of our results and applied inverse probability of censoring weights to adjust for potential selection bias.

A few limitations are worth mentioning. We do not have data on maternal BLLs during pregnancy, which would have allowed us to consider the contribution of prenatal exposure, which has been shown to alter immune function later in life (52, 53, 71). Due to limited funding, we could not measure antibody titers for each vaccine that South African children are scheduled to receive by 3.5 years of age. Instead, we selected representative targets of three classes of vaccine: tetanus (adjuvanted protein), measles (live-attenuated virus), and haemophilus influenzae type b (conjugated polysaccharide-subunit). Although the VHEMBE population is similar to many rural African populations in terms of sociodemographics and lifestyle, it is unclear if our results may be generalizable to populations from urban areas or high-income countries.

In summary, we found inverse associations between exposure to lead and antibody responses to vaccines among South African children. While lead exposure has decreased in many countries

following its removal from gasoline (72), exposure from mining operations, paint, glazes and other sources continue to contribute to lead exposure in some populations (73). Although the CDC has recently revised the BLL reference from 10 to 5 µg/dL (28), our study suggests that BLLs below this cutoff may be associated with a decreased immune response to vaccines. Given that an effective vaccine-induced response requires the proper interaction of multiple cells and pathways, these results may have broader implications for immune response to infection. This hypothesis is supported by data from the National Health and Nutrition Examination Survey (NHANES), which reported associations between BLL and increased seroprevalence for hepatitis B virus, *Helicobacter pylori* and *Toxoplasma gondii* (74). The potential contribution of lead to reduced vaccine efficacy and infections is a serious public health concern, particularly in Sub-Saharan Africa where infectious diseases represent a large proportion of the disease burden (75, 76). Future studies should determine if lead exposure is associated with an increased frequency of childhood infections and whether the associations that we reported with immune response to vaccines persist at later ages.

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Supplemental Material

Figure S1. Bivariate associations between (log_{10}-transformed) lead concentrations and (log_{10}-transformed) measles, tetanus and Hib IgG titers using locally-weighted smoothing scatterplots.

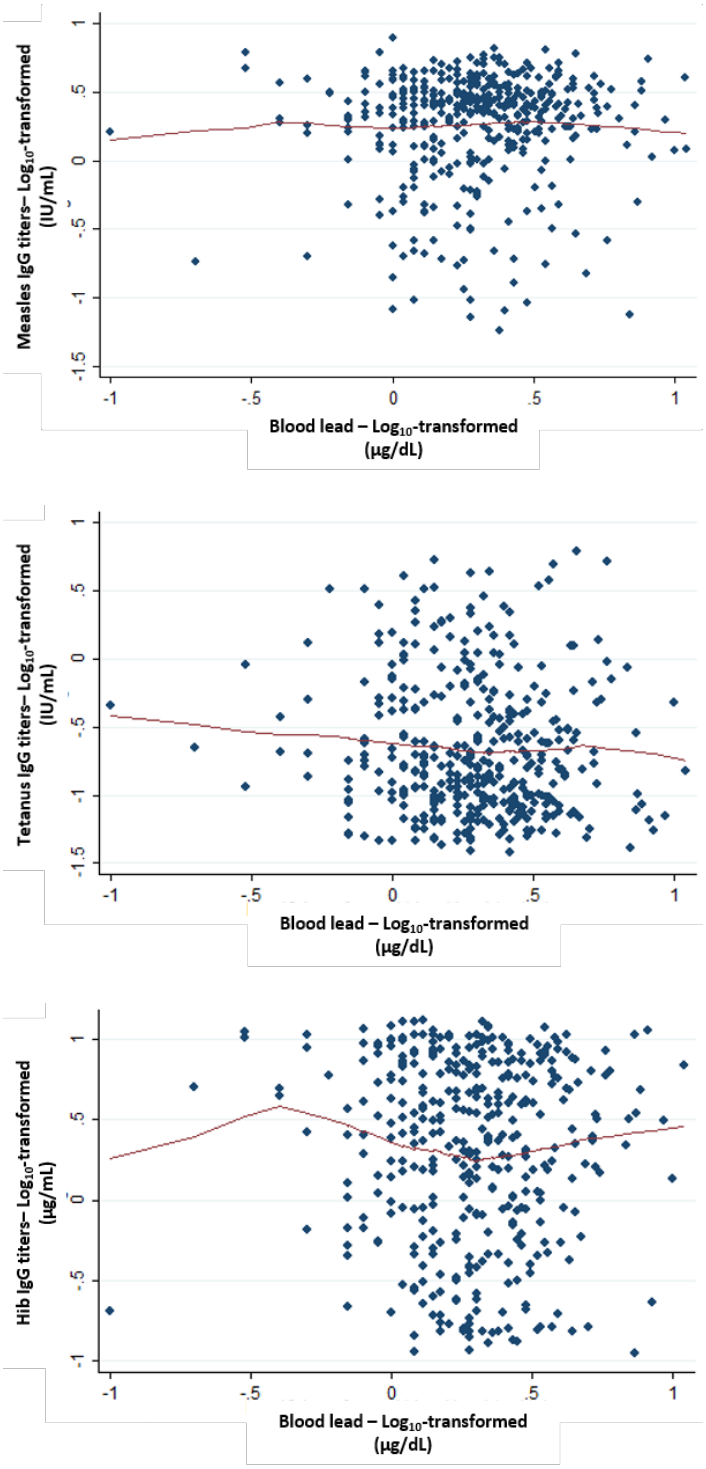

Table S1. Associations between blood lead concentrations at 1 year and vaccine-specific IgG titers at 3.5 years among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa using quantile regression.

	Percentile	β%	95% CI
Measles	25th	2.33	(-42.46, 81.97)
	33rd	34.90	(-6.67, 104.17)
Tetanus	25th	-33.93*	(-53.22, 7.15)
	33rd	-29.20	(-54.29, 73.78)
Hib	25th	12.20	(-47.52, 139.88)
	33rd	9.64	(-54.29, 157.04)

*p<0.05

Models adjusted for maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine

Table S2. Associations between blood lead concentrations at 1 year and relative risk of vaccine-specific IgG titers being below protective level (high cutoff) at 3.5 years among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa.

	Cut-off value	No. below cut-off (%)	RR	95% CI
Measles	1 IU/mL	78 (18)	0.62	(0.31, 1.24)
Tetanus	0.125 IU/mL	152 (39)	1.42	(0.89, 2.28)
Hib	1.5 ug/mL	145 (37)	1.02	(0.61, 1.72)

		Measles RR (95% CI)	Tetanus RR (95% CI)	Hib RR (95% CI)
Child Sex	Male	0.46 (0.19, 1.14)	1.43 (0.76, 2.68)	0.77 (0.39 -1.51)
	Female	0.91 (0.33, 2.47)	1.43 (0.71, 2.86)	1.44 (0.72, 2.88)
Maternal HIV status	Negative	0.94 (0.45, 2.00)‡	1.45 (0.88, 2.38)	0.88 (0.51, 1.52) †
	Positive	0.12 (0.05, 0.28)	1.23 (0.29, 5.24)	2.66 (0.79, 8.97)
Stunting	No	0.85 (0.36, 2.00)*	1.31 (0.76, 2.28)	1.15 (0.65, 2.02)
	Yes	0.26 (0.10, 0.69)	2.00 (0.80, 5.00)	0.69 (0.27, 1.78)

*p_{int}<0.1; †p_{int}<0.05; ‡p_{int}<0.01

Models adjusted for maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine

Table S3. Associations between blood lead concentrations at 1 year and relative risk of vaccine-specific IgG titers being below protective level (low cutoff) at 3.5 years among children participating in the Venda Health Examination of Mothers, Babies and their Environment (VHEMBE), Limpopo, South Africa

	Cut-off value	No. below cut-off (%)	RR	95% CI
Measles	0.2 IU/mL	38 (4.5)	0.91	(0.12, 6.76)
Tetanus	0.08 IU/mL	80 (21)	2.06	(0.98, 4.33)
Hib	0.75 ug/mL	99 (25)	0.94	(0.49, 1.80)

		Measles RR (95% CI)	Tetanus RR (95% CI)	Hib RR (95% CI)
Child Sex	Male	0.19 (0.04, 0.99)†	1.76 (0.63, 4.96)	0.82 (0.35, 1.89)
	Female	8.24 (0.74, 91.92)	2.42 (0.86, 6.77)	1.17 (0.49, 2.82)
Maternal HIV status	Negative	2.76 (0.33, 22.95)*	2.43 (1.09, 5.41)	0.74 (0.38, 1.46)*
	Positive	0.18 (0.03, 0.96)	0.55 (0.10, 2.96)	3.65 (0.78, 17.03)
Stunting	No	5.88 (0.77, 44.87)‡	2.31 (0.98, 5.45)	1.11 (0.54, 2.29)
	Yes	0.14 (0.04, 0.51)	1.10 (0.37, 3.24)	0.69 (0.26, 1.86)

*$p_{int}<0.1$; †$p_{int}<0.05$; ‡$p_{int}<0.01$

Models adjusted for maternal age, HIV status and *p,p'*-DDT serum concentrations, duration of breast feeding and time since last vaccine