Elsevier Editorial System(tm) for Vaccine
Manuscript Draft

Manuscript Number: JVAC-D-15-01437R3

Title: Delayed BCG immunization does not alter antibody responses to EPI vaccines in HIV-exposed and -unexposed South African infants

Article Type: Regular Research Article

Keywords: BCG; humoral immunity; infants; HIV-exposed; South Africa

Corresponding Author: Dr. Heather Jaspan, MD,PhD

Corresponding Author's Institution: University of Cape Town

First Author: Anneke C Hesseling

Order of Authors: Anneke C Hesseling; Anna K Blakney; Christine E Jones; Monika M Esser; Corena de Beer; Louise Kuhn; Mark F Cotton; Heather Jaspan, MD,PhD

Abstract: Background
Bacille Calmette-Guérin (BCG) is routinely given at birth in tuberculosis-endemic settings due to its protective effect against disseminated tuberculosis in infants. BCG is however contraindicated in HIV-infected infants. We investigated whether delaying BCG vaccination to 14 weeks of age affected vaccine-induced antibody responses to Haemophilus influenza type b (Hib)-conjugate, pertussis, tetanus and Hepatitis B (HBV) vaccines, in HIV-exposed uninfected (HEU) and -unexposed uninfected (HUU) infants.
Methods
Infants were randomized to receive BCG at birth or at 14 weeks of age. Blood was taken at 14, 24, and 52 weeks of age and analyzed for Hib, pertussis, tetanus and HBV specific antibodies.
Results
BCG was given either at birth (106 infants, 51 HEU) or at 14 weeks of age (74 infants, 50 HEU). The timing of BCG vaccination did not influence the antibody response to any antigen studied. However, in a non-randomised comparison, HEU infants had higher Hib antibody concentrations at weeks 14 and 24 ($p=0.001$ and $<0.001$ respectively) and pertussis at week 24 ($p=0.003$). Conversely, HEU infants had lower antibody concentrations to HBV at 14 and 52 weeks ($p=0.032$ and $p=0.031$) with no differences in tetanus titres.
Conclusions
HIV exposure, but not the timing of BCG vaccination, was associated with antibody concentrations to Hib, pertussis, HBV and tetanus primary immunization.
Clinical Trial Registration: DOH-27-1106-1520

**Highlights**:

- Timing of BCG vaccination did not influence antibody levels to Hib, pertussis, tetanus or HBV
- Effects of early versus late BCG vaccination did not differ between HIV-exposed and –unexposed groups
- HIV-exposure without infection was associated with increased Hib and pertussis antibody concentrations

# ~~Timing of~~Delayed BCG immunization does not alter antibody responses to EPI vaccines in HIV-exposed ~~, uninfected~~ and -unexposed South African infants

Anneke C. Hesseling[1,*], Anna K. Blakney[2,3], Christine E. Jones[2,4, 5], Monika M. Esser[6], Corena de Beer[7], Louise Kuhn[8], Mark F. Cotton[9**] and Heather B. Jaspan[2,10**]

1. Desmond Tutu TB Centre, Department of Paediatrics and Child Health, Faculty of Medicine and Health Sciences, Stellenbosch University, 2. Division of Immunology, Institute of Infectious Disease and Molecular Medicine and Clinical Laboratory Sciences, University of Cape Town, 3. Department of Bioengineering, University of Washington, Seattle, WA, 4. Paediatric Infectious Diseases Research Group, Institute for Infection and Immunity, St. George's, University of London, Cranmer Terrace, London SW17 0RE, UK, 5. Department of Academic Paediatrics, Imperial College London, Norfolk Place, London W2 1NY, UK, 6. Immunology Unit , Division of Medical Microbiology, Department of Pathology, National Health Laboratory Service, University of Stellenbosch, Cape Town, South Africa, 7. Division of Medical Virology, Department of Pathology, National Health Laboratory Service, Stellenbosch University, 8. Gertrude H. Sergievsky Center, College of Physicians and Surgeons, and Department of Epidemiology, Mailman School of Public Health, New York, New York, USA 9. Children's Infectious Diseases Clinical Research Unit, Faculty of Medicine and Health Sciences, Stellenbosch University, Tygerberg, South Africa, 10. Seattle Children's Research Institute and Departments of Pediatrics and Global Health, University of Washington, Seattle, WA,

** contributed equally

*** Corresponding author information**:

Desmond Tutu TB Centre, Department of Paediatrics and Child Health, Faculty of Medicine and Health Sciences, Stellenbosch University PO Box 19063, Tygerberg, 7505, South Africa

Phone +27 (0) 21 9389173

Fax +27 (0) 21 9389719

Email: annekeh@sun.ac.za

## ABSTRACT

**Abstract word count**: 208

### Background

Bacille Calmette-Guérin (BCG) is routinely given at birth in tuberculosis-endemic settings due to its protective effect against disseminated tuberculosis in infants. BCG is however contraindicated in HIV-infected infants. We investigated whether delaying BCG vaccination to 14 weeks of age affected vaccine-induced antibody responses to *Haemophilus influenza* type b (Hib)-conjugate, pertussis, tetanus and Hepatitis B (HBV) vaccines, in HIV-exposed uninfected (HEU) and -unexposed uninfected (HUU) infants.

### Methods

Infants were randomized to receive BCG at birth or at 14 weeks of age. Blood was taken at 14, 24, and 52 weeks of age and analyzed for Hib, pertussis, tetanus and HBV specific antibodies.

### Results

BCG was given either at birth (106 infants, 51 HEU) or at 14 weeks of age (74 infants, 50 HEU). The timing of BCG vaccination did not influence the antibody response to any antigen studied. However, in a non-randomised comparison, HEU infants had higher Hib antibody concentrations at weeks 14 and 24 ($p=0.001$ and $<0.001$ respectively) and pertussis at week 24 ($p=0.003$). Conversely, HEU infants had lower antibody concentrations to HBV at 14 and 52 weeks ($p=0.032$ and $p=0.031$) with no differences in tetanus titres.

### Conclusions

HIV exposure, but not the timing of BCG vaccination, was associated with antibody concentrations to Hib, pertussis, HBV and tetanus primary immunization.

**Clinical Trial Registration:** DOH-27-1106-1520

**Key words**: BCG, humoral immunity, infants, HIV-exposed, South Africa

## BACKGROUND

Approximately 1.4 million children under five years of age die annually of vaccine preventable infectious diseases.[1] Maternal and paediatric HIV infections contribute considerably to the burden of infectious disease in developing countries, and HIV-exposed infants constitute up to 30% of infants born in the public health sector in settings with high burden of HIV such as South Africa.[2] Studies of vaccine responsiveness amongst this subpopulation are limited but are important considering their high morbidity and mortality, even in the absence of infant HIV infection. [3-7]

Many vaccines rely on inducing antibodies to pathogens or their toxins. Although maternal IgG crosses the placenta *in utero* providing some protection against vaccine preventable infections, infant IgG and IgA responses to pathogens remain relatively weak in the first 12 months of life. [8, 9] Despite B-cell priming in neonates generating memory B cells, maternal antibody can inhibit primary humoral responses [6]. Antenatal HIV exposure has been associated with lower specific antibody levels in HIV-exposed uninfected (HEU) infants than HIV-unexposed uninfected infants (HUU) at birth, although subsequent responses in HEU infants to certain vaccines may be better than HUU infants, perhaps due to less inhibition.[7] The development of vaccines or vaccination strategies to induce early protective immune responses in infants is a major challenge in vaccinology.

BCG induces robust Th-1 type cellular immune responses, even at birth.[10-12] Apart from its protective effect against disseminated tuberculosis (TB) in young children, [13] BCG is associated with other "non-specific" protective effects and decreases non-TB-related child morbidity and mortality in settings with high background rates of infectious morbidity.[14, 15] For example, trials from West Africa showed that BCG reduces neonatal mortality by more than 40%, mainly by preventing neonatal sepsis and respiratory infections.[16, 17] In

addition, Ota et al found that BCG enhanced humoral immunity to oral polio and hepatitis B vaccinations but not to tetanus and diphtheria toxoids, while de Castro et al found that BCG vaccination at birth may decrease hospitalization due to respiratory infection and sepsis, due to heterologous protection. [12, 18]

In addition to adding new vaccines to the Expanded Programme on Immunization (EPI) schedule, it is necessary to assess potential vaccine interactions as well as specific and non-specific vaccine effects of existing vaccines, with consideration of maternal HIV status. [19] BCG-related effects on unrelated vaccine-induced antibody responses could be important in settings with high HIV prevalence, where BCG is still routinely given at birth. Based on concerns of BCG vaccine safety in HIV-infected infants, delaying BCG vaccination in HIV-exposed infants until HIV has been excluded, is a relevant strategy, but its timing could influence vaccine responsiveness. [20]

We investigated the effect of delayed BCG vaccination on antibody responses to Hib-conjugate, whole cell *Bordetella pertussis* (wP), tetanus toxoid (TT) and hepatitis B (HBV) vaccines, in HEU and HUU infants. We hypothesized that BCG vaccination at birth would increase antibody responses to other vaccines through induction of non-specific immunological effects, and that these responses would be more pronounced in HIV-exposed infants.

## METHODS

### Study setting

This study was conducted from 1 April 2006 to 31 March 2008 at the community-based Site B Midwife Obstetric Unit and well baby clinic in Khayelitsha, Cape Town, Western Cape Province, South Africa, where the maternal HIV prevalence was 32.7 % (95% CI: 29.5-

35.9%) in 2008 [21] with a well-established prevention of mother to child HIV transmission (PMTCT) program.

During the study, the South African EPI recommended intradermal BCG vaccination (0.05 ml reconstituted vaccine, Danish strain BCG, Statens Serum Institute, 1331) and oral live polio (OPV, Sabin, Sanofi Pasteur, France, 2 drops orally) at birth. Diphtheria and TT vaccines, wP and Hib conjugate vaccines (PRP-T) administered as DTP-Hib (TETRActHib,™ Sanofi Pasteur, France, 0.5ml intramuscular) were recommended at 6, 10 and 14 weeks of age, and were co-administered with Hepatitis B (HBV; Heberbiotec, Cuba, 0.5ml; intramuscular) and OPV. Live measles vaccine (Sanofi Pasteur, France, 0.5ml intramuscular) was recommended at 9 months. As per South African guidelines, pregnant women did not receive anti-tetanus vaccination. BCG coverage was estimated at 99% during 2005.[22]

**Eligibility and randomization**

Recruitment, enrolment, BCG vaccination and surveillance were carried out as previously described[23]. Briefly, this was an individual, single-blinded, exploratory randomized Phase 2 clinical trial investigating immunological effects of early versus delayed BCG vaccination in HIV-exposed and –unexposed infants (DOH-27-1106-1520). Pregnant HIV-positive and –negative women were recruited at the midwife obstetric unit.. Enrolment was stratified by maternal HIV status to ensure that two-thirds of infants (n=120) were HIV-exposed and one-third (n=60) HIV-unexposed (control group), using 2 separate randomization lists. Infants were randomized to BCG, given intradermally in the right deltoid area, at birth (routine BCG), versus at 14 weeks of age (delayed BCG). The concealed envelope method was used by the study nurse, who enrolled participants antenatally, and was blinded to study allocation. Stratified randomization (2 separate lists for HIV-infected and uninfected women) was completed by an independent statistician, using randomization with blocks

varying in size from 2 to 6 with random ordering. Study nurses who assessed and followed infants were not blinded to treatment allocation.

Women with pregnancies of estimated 32 or more weeks gestation were screened for eligibility after routine testing for HIV and written informed consent was obtained. The following were postnatal infant exclusion criteria: stillbirth, birth weight <1.6 kg, severe congenital malformation, asphyxia or other severe illness at birth since under these circumstances, BCG is not routinely given in South Africa. Protocol violators were defined as infants randomized to receive delayed BCG vaccination at 14 weeks, but who were inadvertently given BCG at birth by routine personnel.

**Study measures and follow-up**

Infant HIV testing and clinical follow-up were performed as previously described.[23] Briefly, a single infant HIV DNA polymerase chain reaction test (Amplicor, Roche Molecular Diagnostics, Pleasanton , CA) was routinely offered at 6 and at 14 weeks of age. The routine testing algorithm at the time recommended testing at 14 weeks of age only. Follow-up was carried out on the same premises, at the Site B well baby clinic, where infants would have attended routine clinical care. Antibody levels were classified using the following accepted measures. Anti-Hib capsular polysaccharide (PRP-T) IgG antibodies were measured using the VaccZyme™ Human Anti Hib Enzyme Immunoassay kit (MK016, The Binding Site Ltd, Birmingham, England). Measurement of specific IgG antibodies to *Bordetella pertussis* was completed using pertussis toxin (PT) and filamentous hemagglutinin (FHA) as antigen preparation using the SERION ELISA classic kit (Serion Immundiagnostica GmbH, Würzburg, Germany). Anti-tetanus IgG was measured with the SERION ELISA *classic* kit (Serion Immundiagnostica GmbH, Würzburg, Germany). Specific IgG against Hepatitis B surface antigen (HBsAg) were quantified using a semi-automated ELISA method (AxSym

HBsAb, Abbott Diagnostics, measuring range: 2-1000 mIU/ml). Hib antibody level was classified as protective if >1 mg/ml and non-protective level if ≤1 mg/ml.[24] Since there is no correlate of protection against pertussis, anti-pertussis antibody was classified positive if >30 FDA-U/ml, indeterminate if 20-30F FDA-U/ml and negative if <20 FDA-U/ml, as defined by the manufacturer.[25] Tetanus antibodies were classified as having no immunity if <0.01 IU/ml; no safe immunity if 0.01-0.1 IU/ml; sufficient immunity if 0.11-5.0 IU/ml and long-term immunity: >5.0 IU/ml, and sufficient or long-term immunity were considered protection for the purposes of analysis.[26] HBsAb concentrations <10 mIU/ml were classified as negative and when ≥ 10 mIU/ml as positive, based on standard international criteria. [27]

In a subset of participants (n=38, with 17 HEU and 21 HUU infants) with sufficient additional stored sera, the relative avidity of IgG against Hib capsular polysaccharide was measured to assess qualitative response (VaccZyme Hib ELISA accessory pack, The Binding Site Ltd, Birmingham, U.K.). Typical reported values range from 26.4- 68.3 mg/ml.

**Statistical analysis**

The primary end point was the difference in the magnitude of antibody responses to Hib, pertussis, HBV and TT between infants in the early and the delayed BCG groups at 14 weeks of age. Secondly, the effect of HIV exposure on vaccine antibody titres was investigated at week 14, 24 and 52. Sample size estimates for this exploratory study were calculated assuming a 30% difference in vaccine antibody responses between BCG vaccinated and unvaccinated infants, and a 30% difference between HIV-exposed and unexposed infants, at week 14. HIV-infected infants (n=2) were excluded from analysis.

The effect of timing of BCG vaccination and HIV exposure on the proportion of responders was compared using the Chi-squared test or Fisher's exact where appropriate; effect estimates (OR; 95% CI) were calculated. Log-transformed data were used to compare mean antibody titres. Antibody concentrations of zero or below the assay cutoff were given an arbitrary value of half the cutoff for geometric mean concentration (GMC) calculation. The 2-sided t test was used for comparison of GMC antibody values.

A base-10 linear regression model with log-transformed values was used to compare the proportion of "protective" responses to Hib, pertussis, HBV and TT vaccines at week 14 using BCG vaccination as primary exposure variable. Other variables included were either specified in the study hypothesis and included HIV exposure, or were known to influence vaccine antibody responses such as sex and birth weight [28-30]. Statistical significance was inferred at the 2-sided 0.05 level. SPSS software (version 16.0, Chicago, Ill) was used for analyses. Missing values were excluded from analysis. The CONSORT guidelines were used for reporting. [31] The Stellenbosch University Human Research Ethics Committee approved the study (trial number DOH-27-1106-1520).

**RESULTS**

120 HIV-infected and 60 HIV-uninfected women were enrolled during pregnancy. Following randomization, 5 infants (2.8%) were excluded; 1 mother withdrew from the study due to geographic relocation and 4 HIV-exposed infants were stillborn. These infants were replaced with additional randomized participants..

Figure 1 provides an overview of the study cohort, per protocol. Randomization of infants resulted in balance for maternal HIV status, infant sex, mean birth weight, maternal CD4+ T cell count and maternal HAART (Table 1). The mean birth weight overall was 3206 grams

(standard deviation; SD: 44). Only 6 infants (3.33%) had birth weight <2500 grams. The mean maternal CD4+ T lymphocyte amongst HIV-infected women was 363 cells/$mm^3$ (SD 23.7). Of HIV-infected women, 27 (15%) had a CD4+ T lymphocyte count ≤200 cells/$mm^3$; these women were all on highly active antiretroviral therapy (HAART; Zidovudine, Lamivudine and Nevirapine).

Of the 90 infants randomized to delayed BCG, 16 were inadvertently given immediate BCG by routine labour ward personnel, resulting in 106 infants in the birth (67, 63.2% HIV-exposed) and 74 infants (46, 62.2% HIV-exposed) in the delayed BCG groups, respectively. Analysis was therefore based on actual vaccination (per protocol) status.

**Proportion of infants with positive/protective antibody concentrations**

Proportions of infants with "protective" antibody concentrations to Hib, pertussis, TT and HBV are reflected in Supplementary Table 2. In general, the proportion of infants with "protective" Hib and pertussis titres was low at all timepoints. Only 62% of infants had antibody concentrations to Hib correlating with protective immunity at week 24, following verified completion of weeks 6, 10 and 14 vaccinations; the level declined to 35% at week 52. At week 24, 48% of infants had positive pertussis titres; 45% of infants maintained positive titres at week 52. In contrast, the proportion of infants with positive/protective titres to TT and HBV vaccines was high with all infants having antibody concentrations correlating with sufficient or long-term protective immunity to tetanus vaccine at week 24; positive responses to HBV were detected in all infants. Only 1.2% of infants lacked protective response to TT at week 52.

**Effect of BCG vaccination timing on antibody concentrations**

Overall, there was no detectable effect of BCG vaccination timing on the GMC antibody titres to Hib, pertussis, or TT at weeks 14, 24 or 52 (Figure 2). There was a trend for higher antibody responses to HBV in the birth BCG group at week 14 (GMC in birth group: 30.5 vs. 10.1in the delayed group; p=0.090). Similarly, there was no observed effect of BCG on the proportion of infants with "protective" antibody concentrations (Supplementary Table 1). Results were similar comparing the proportion of infants with "protective" responses in the birth and delayed BCG groups at 14 weeks when infants in the protocol violator group were excluded (data not shown).

**Effect of *in utero* HIV exposure on antibody concentrations**

Stratified analysis of antibody titres between HIV-exposed and unexposed infants produced similar results in both the birth and delayed BCG groups (data not shown); the birth and delayed groups were therefore combined for further analysis. In the combined analysis, HIV-exposed infants consistently demonstrated higher GMC antibody titres to Hib at weeks 14 and 24, and also to pertussis at week 24 (Figure 3a-b). These higher concentrations corresponded to a higher proportion of HIV-exposed infants with positive/protective antibody concentrations to Hib at week 14 (OR: 1.75; 95% CI: 1.17-2.63, p=0.007) and week 24 (OR: 2.09; 95% CI: 1.37-3.17, p=0.001) and to pertussis at week 24 (OR: 1.80; 95% CI: 1.19-2.72, p=0.007) (Table 2). Conversely, HIV-exposed infants had significantly lower GMC of HBV antibody at week 14 and week 52, although all were above positive/protective concentrations (Figure 3d). Antibody concentrations to tetanus vaccine were similar between HIV-exposed and unexposed infants (Figure 3c).

**Effect of BCG vaccination timing on antibody concentrations in HIV-exposed infants**

Because delaying BCG vaccination until 14 weeks of age would be most relevant for infants born to HIV-positive mothers, we compared the effects of BCG vaccination at birth versus 14 weeks of age in HIV-exposed infants. There was no detectable effect of BCG vaccination on the GMC of antibody to Hib, pertussis, tetanus of HBV at weeks 14, 24, or 52 (Supplementary Figure 1).

**Predictors of antibody titres against against Hib and pertussis**

In a multivariable ~~base-10~~ linear regression model with log10-transformed values for factors ~~(including BCG vaccination as primary predictor and HIV exposure status, birth weight and sex as covariates)~~ associated with "protective" anti-Hib concentration at 14 weeks of age (including BCG vaccination as primary predictor and HIV exposure status, birth weight and sex as covariates), only HIV exposure remained associated with higher concentrations (coefficient 0.47; 95% CI: 0.21-0.72; p<0.001; adjusted $R^2$=0.101) (Table 3); the timing of BCG vaccination was not a predictor. In a similar model for pertussis, none of the predictors were associated with "protection" against pertussis at week 14 (adjusted $R^2$=0.020; Table 3). For ~~both~~the Hib linear regression model~~s~~, a plot of the residuals versus the antibody titres for Hib ~~and pertussis~~ were randomly distributed, indicating that a linear regression model was appropriate for analysis. For pertussis, a plot of the residuals versus the antibody titres revealed a single outlier; removal of this single outlier did not alter the model. Linear regression analyses of predictors for positive responses to TT or HBV were not completed since all infants had sufficient or long-term immunity to TT, and 99.2% immunity HBV at week 14, respectively.

Formatted: Superscript

Formatted: Superscript

Formatted: Not Superscript/ Subscript

**Qualitative Hib antibody**

Qualitative analyses for Hib were completed in 38 infants with available serum samples, in 17 HEU and in 21 HUU infants. The mean binding index was 72.06 (SD 45.67) classified as "high or adequate". There was no difference between the mean index in HEU and HUU infants (mean: 66.01; SD: 18.41 in HEU vs. 76.95; SD: 59.42 in HUU infants; p=0.470).

**DISCUSSION**

In this exploratory randomized controlled trial, the timing of BCG vaccination did not alter antibody responses to Hib, pertussis, HBV and TT vaccines in HEU or HUU infants. Despite a trend for increased antibody responses to HBV, there was no evidence for BCG-induced increased HBV antibody concentrations, both when responses were measured at 14 weeks (comparing the effect of BCG given at birth to infants who had not yet received BCG), and at 24 and 52 weeks (comparing the effect of BCG given at birth to BCG given at 14 weeks). In a non-randomised comparison, we found that HIV-exposed infants had significantly higher antibody concentrations to pertussis and Hib than HIV-unexposed infants, following vaccination. This study is limited by the lack of data on maternal and baseline (birth) infant antibody responses, and a lack of correlation with clinical endpoints. The protocol violations (vaccination with BCG when subjects were randomized to the delayed BCG group) and high loss to follow-up may also have introduced bias. We did not find any differences at birth in sex, birth weight, or maternal HIV status between infants in the protocol deviation group, and infants who were vaccinated as per randomisation schedule, but this does not exclude bias (for example, if loss to follow-up was associated with differences in response to immunization).

We found that the proportion of children with Hib antibody >1 mg/ml were consistently of greater magnitude amongst HEU compared to HUU infants in both the early and delayed vaccination groups and remained significant after controlling for the timing of BCG vaccination, birth weight and sex, at week 14. The proportion of children with anti-pertussis antibody >30 FDA-U/ml at 24 weeks of age was also higher amongst HIV-exposed infants. These data are consistent with our own and other published data.[5, 23] The higher observed antibody responses to Hib and pertussis amongst HEU infants may be due to reduced maternal-infant placental antibody transfer in the presence of maternal HIV infection. Jones et al showed that HEU infants had lower concentrations of specific antibodies at birth than HUU infants to Hib, wP, pneumococcus and tetanus vaccines, in the identical study setting.[7] Maternal antibodies can inhibit infant responses to measles, tetanus, wP, and Hib vaccines, although this effect varies considerably between different vaccines and studies.[32-34] Another explanation for this may be non-specific T cell activation as a result of *in utero*, peripartum or postpartum HIV exposure and increased immune maturity.[35]

Maternal infections, including HIV, can negatively impact placental integrity and maternal-foetal antibody transfer. A study amongst Kenyan HIV-infected women and their infants showed that high maternal viral load was associated with reduced transplacental transfer of measles antibodies.[36] Although vaccination of women during pregnancy leads to transplacental transfer of antibodies, high total maternal IgG concentrations may lead to decreased antibody concentrations in infants; this effect can last until up to 12 months of age.[37] In this study, we have no data on prevaccination antibody levels, and therefore cannot distinguish whether HEU infants had poor antibody responses due to inherent deficiencies in immunity, or due to differences in passive maternal antibody levels. Although persistence of maternal antibodies may limit infant antibody responses, priming

of infant T-cell responses are unaffected by these passively transferred antibodies, which may explain why humoral responses are altered, but not cellular responses [37, 38]. In this study, we did not measure T cell responses to vaccines, but we found compromised IFN-γ ELISPOT responses in HEU.[23] Antibodies alone are a convenient yet imperfect measure of vaccine-induced effects, as cell-mediated immunity is likely also an important component of protection against vaccine-preventable disease.

Our finding that BCG timing had no effect on antibody responses to unrelated vaccines is inconsistent with data from a single study from Ota et al from the Gambia, a setting with high infant mortality but low HIV prevalence. Here, giving BCG at birth markedly increased the cellular and antibody responses to HBV in low birth weight infants at 18 weeks. [12] BCG enhanced antibody responses to OPV, but only when given at the time of boosting. BCG had no detectable effect on antibody responses to TT (consistent with our findings) and diphtheria toxoid vaccines at 18 weeks. A proposed mechanism for these BCG effects on HBV and OPV responses is the enhanced activation of T lymphocytes by dendritic cells and BCG- enhanced induction of memory B cells. Unlike in the Gambia, infants in South Africa receive OPV but not HBV at birth. This may partly explain the lack of effect of BCG on response to HBV in our study. However, given the almost universally protective immunity elicited by HBV vaccine in infants, the clinical relevance of changes in responsiveness due to concomitant BCG vaccination is unknown.

The low proportion of infants in our study with “protective” antibody to Hib and pertussis at 24 weeks, 10 weeks after vaccination at week 14, is concerning. Low responses confirm that boosting is essential, as already practiced in the EPI. However, the coverage of DTP-Hib 4 boosting at 18 months was low (<60%) ii routine care at the time of the study, which leaves a large gap in “protective” immunity. Hussey et al reported similar low levels of

protective antibody responses (69.1%) following vaccination with the same Hib vaccine amongst South African infants at 18 weeks of age in an adjacent community.[38] A recent outbreak of pertussis in Bloemfontein, South Africa, confirms the need for better immunization practices and suggests that young children are vulnerable and can contribute to its spread [39].

Our findings suggest that the timing of BCG vaccination does not have a major effect on the antibody responses to Hib, pertussis, tetanus and hepatitis B vaccines amongst South African infants; however, delaying BCG vaccination to 14 weeks of age may deny children the possible beneficial non-specific reductions in pneumonia and sepsis after BCG at birth, [16, 17] and it may affect cell-mediated responses to other vaccines and alter the clinical effects (neither of which were assessed in this study). Although antibody responses to Hib and pertussis were higher amongst HIV-exposed than HIV-unexposed infants, they were not "protective" in a third of infants by 52 weeks of age. These findings support further investigation into optimizing infant vaccination strategies in settings with high HIV prevalence.

**ACKNOWLEDGEMENTS**

We would like to thank the clinical team including Barbara Magwegwe, Nonkonzo Dziba, Eunice Mhlamba and Stanzi Le Roux. We thank the facility managers and staff at the Site B midwife obstetric unit and comprehensive community clinic for their support. Funding for this study was through a Thrasher Research Fund award [NR-0006] and an Elizabeth Glazer Pediatrics Aids Foundation international Leadership award Sub Grant ZQ-00-8-520-0123-0-00 and a British Commonwealth Scholarship to ACH and a National Science Foundation Graduate Research Fellowship to AKB.

## CONFLICT OF INTEREST

All authors: none declare

## REFERENCES

[1] WHO. Immunization surveillance, assessment and monitoring. 2008. Available at http://www.who.int/immunization/monitoring_surveillance/burden/estimates/en. Accessed 6 December 2015.

[2] Dorrington RE, Bourne D. Re-estimated provincial HIV antenatal survey prevalence for 2007 and a reinterpretation of the national trend. S Afr Med J 2008;98:940-1.

[3] Taha TE, Graham SM, Kumwenda NI, Broadhead RL, Hoover DR, Markakis D, et al. Morbidity among human immunodeficiency virus-1-infected and -uninfected African children. Pediatrics 2000;106:E77.

[4] Spira R, Lepage P, Msellati P, Van De Perre P, Leroy V, Simonon A, et al. Natural history of human immunodeficiency virus type 1 infection in children: a five-year prospective study in Rwanda. Mother-to-Child HIV-1 Transmission Study Group. Pediatrics 1999;104:E56.

401 [5] Reikie BA, Naidoo S, Ruck CE, Slogrove AL, de Beer C, la Grange H, et al. Antibody
402 responses to vaccination among South African HIV-exposed and unexposed uninfected
403 infants during the first 2 years of life. Clin Vaccine Immunol 2013;20:33-8.
404 [6] Jones CE, Hesseling AC, Tena-Coki NG, Scriba TJ, Chegou NN, Kidd M, et al. The impact of
405 HIV exposure and maternal Mycobacterium tuberculosis infection on infant immune
406 responses to bacille Calmette-Guérin vaccination. AIDS 2015;29:155.
407 [7] Jones CE, Naidoo S, Beer DC, Esser M, Kampmann B, Hesseling AC. Maternal HIV
408 infection and antibody responses against vaccine-preventable diseases in uninfected
409 infants. JAMA 2011;305:576-84.
410 [8] Siegrist C. The challenges of vaccine responses in early life: selected examples. J Comp
411 Pathol 2007; 137 Suppl 1:S4-9..
412 [9] Siegrist CA. Neonatal and early life vaccinology. Vaccine 2001 ;19(25-26):3331-46.
413 [10] Kagina B, M. N., Abel B, Bowmaker M, Scriba TJ, Gelderbloem S, Smit E, et al. Delaying
414 BCG vaccination from birth to 10 weeks of age may result in an enhanced memory CD4 T
415 cell response. Vaccine 2009;27:5488-95.
416 [11] Marchant A, Goetgheuber T, Ota M, Wolfe I, Ceesay S, De Groote D, et al. Newborns
417 develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guerin
418 vaccination. J Immunol 1999;163:2249-55.
419 [12] Ota M, Vekemans J. Influence of Mycobacterium bovis bacillus Calmette-Guerin on
420 antibody and cytokine responses to human neonatal vaccination. J Immunol 2002;168:919-
421 25.
422 [13] Trunz B, Fine P, Dye C et al. Effect of BCG vaccination on childhood tuberculous
423 meningitis and miliary tuberculosis worldwide: a meta-analysis and assessment of cost-
424 effectiveness. Lancet. 2006;367:1173-80.

425 [14] Garly ML, Martins CL, Balé C, Baldé MA, Hedegaard KL, Gustafson P, et al. BCG scar and
426 positive tuberculin reaction associated with reduced child mortality in West Africa: a non-
427 specific beneficial effect of BCG? Vaccine 2003; 21:2782–90.
428 [15] Roth AE, Stensballe LG, Garly ML, Aaby P. Beneficial non-targeted effects of BCG—
429 ethical implications for the coming introduction of new TB vaccines. Tuberculosis
430 2006;86:397-403.
431 [16] Aaby P, Roth A, Ravn H, Napirna BM. Randomized trial of BCG vaccination at birth to
432 low-birth-weight children: beneficial nonspecific effects in the neonatal period? J Infect Dis
433 2011;204:245-52.
434 [17] Biering-Sørensen S, Aaby P, Napirna BM. Small randomized trial among low–birth-
435 weight children receiving Bacillus Calmette-Guérin vaccination at first health center contact.
436 Pediatr Infect Dis J 2012;31:306-8.
437 [18] de Castro MJ, Pardo-Seco J, Martinon-Torres F. Nonspecific (Heterologous) Protection
438 of Neonatal BCG Vaccination Against Hospitalization Due to Respiratory Infection and
439 Sepsis. Clin Infect Dis 2015;60:1611-9.
440 [19] Fine PEM, Smith PG. Non-specific effects of vaccines-an important analytical insight,
441 and call for a workshop. Trop Med Int Health. 2007;12:1-4.
442 [20] Hesseling AC, Marais BJ, Gie RP, Schaaf HS, Fine PE, Godfrey-Faussett P, et al. The risk of
443 disseminated Bacille Calmette-Guerin (BCG) disease in HIV-infected children. Vaccine.
444 2007;25:14-8.
445 [21] Shaikh N, Abdullah F, Lombard CJ, Smit L, Bradshaw D, Makubalo L. Masking through
446 averages-intraprovincial heterogeneity in HIV prevalence within the Western Cape. S Afr
447 Med J. 2006;96:538-43.
448 [22] Corrigal J, Coetzee D, Cameron N. Is the Western Cape at risk of an outbreak of
449 preventable childhood diseases? Lessons from an evaluation of routine immunisation

coverage. S Afr Med J 2008; 98: 41-45.

[23] Hesseling AC, Jaspan HB, Black GF, Nene N, Walzl G. Immunogenicity of BCG in HIV-exposed and unexposed infants following routine birth or delayed vaccination. Int J Tuberc Lung Dis. 2015;19(4):454-462. .

[24] Kayhty H, Peltola H, Karanko V, Makela PH. The protective level of serum antibodies to the capsular polysaccharide of Haemophilus influenzae type b. J Infect Dis. 1983;147:1100-.

[25] De Melker H, Versteegh FG, Conyn-Van Spaendonck MA, Elvers LH, Berbers GA, van Der Zee A, Schellekens JF. Specificity and sensitivity of high levels of immunoglobulin G antibodies against pertussis toxin in a single serum sample for diagnosis of infection with Bordetella pertussis. J Clin Microbiol. 2000;38:800-6.

[26] Schroder JP, Kuhlmann WD. Tetanus vaccine protection. Dtsch Med Wochenschr. 1993;118: 118(26):1000.

[27] Odinsen O, Owusu-Ofori S, Dompreh A, Sarkodie F, Opare-Sem O, Parker D, et al. Antibody detection and kinetics of antibody production during early stages of immunization with hepatitis B virus vaccine. Clin Vaccine Immunol 2007: 14(12):1623-8..

[28] Baynam G, Zhang G, Khoo SK, Sly P, Holt P, Goldblatt J, Le Souëf PN. Gender-specific effects of cytokine gene polymorphisms on childhood vaccine responses. Vaccine 2008;26:3574-9.

[29] Greenwood B, Bradley-Moore AM, Bradley AK, Kirkwood BR, Gilles HM. The immune response to vaccination in undernourished and well-nourished Nigerian children. Ann Trop Med Parasitol 1986;80:537-44.

[30] Roth A, Jensen H, Garly M-L, Djana Q, Martins CL, Sodemann M, et al. Low birth weight infants and Calmette-Guerin bacillus vaccination at birth: community study from Guinea-Bissau. Pediatr Infect Dis J 2004;23:544-50.

474 [31] Moher D, Hopewell S, Schulz KF, Montori V. CONSORT 2010 explanation and
475 elaboration: updated guidelines for reporting parallel group randomised trials. J Clin
476 Epidemiol. 2010;63:e1-e37.
477 [32] Albrecht P, Ennis FA, Saltzman EJ, Krugman S. Persistence of maternal antibody in
478 infants beyond 12 months: mechanism of measles vaccine failure. J Pediatr 1977;91:715-8.
479 [33] Sarvas H, Kurikka S, Seppälä IJ, Mäkelä PH, Mäkelä O. Maternal antibodies partly inhibit
480 an active antibody response to routine tetanus toxoid immunization in infants. J infect Dis.
481 1992;165:977-9.
482 [34] Siegrist CA. Mechanisms by which maternal antibodies influence infant vaccine
483 responses: review of hypotheses and definition of main determinants. Vaccine. 2003:3406-
484 12.
485 [35] Clerici M, Saresella M, Colombo F, Fossati S, Sala N, Bricalli D, et al. T-lymphocyte
486 maturation abnormalities in uninfected newborns and children with vertical exposure to
487 HIV. Blood 2000;96:3866-72.
488 [36] Farquhar C, Nduati R, Haigwood N, Sutton W, Mbori-Ngacha D, Richardson B, et al. High
489 maternal HIV-1 viral load during pregnancy is associated with reduced placental transfer of
490 measles IgG antibody. J Acquir Immune Defic Syndr 2005;40:494-7.
491 [37] Gans HA, Arvin AM, Galinus J, Logan L, DeHovitz R, Maldonado Y. Deficiency of the
492 humoral immune response to measles vaccine in infants immunized at age 6 months. JAMA.
493 1998;280:527-32.
494 [38] Hussey G, Malan H, Hughes J, Eley B, Piollet M, Charrondiere M, et al. Safety and
495 immunogenicity of TETRActHIB (a vaccine combining DTP vaccine and Haemophilus
496 influenzae type B conjugate vaccine), administered to infants at 6, 10 and 14 weeks of age. S
497 Afr Med J. 2002;92:53-7.

[39] Hallbauer UM, Goosen Y. An outbreak of pertussis in Bloemfontein, South Africa, 2008–2009. S Afr Fam Pract. 2011;53(5):493-500.

**Figure and Table Captions:**

Figure 1: Flowchart of BCG-vaccinated infants investigated for serum antibody levels following a per protocol analysis.

Table 1. Characteristics of 180 infants randomized to BCG at birth or delayed BCG vaccination at 14 weeks of age, by randomization arm.

Figure 2. Concentration of antibody titres to a) Hib, b) pertussis, c) tetanus and d) HBV measured at 14, 24, and 52 weeks of age in infants who received BCG at birth (dark grey dots) vs. infants who received delayed BCG at 14 weeks of age (light grey dots), regardless of HIV-exposure status. Shaded area indicates "protective" antibody concentrations. Bar and error bars represent geometric mean concentration (GMC) with 95% confidence interval.

Figure 3. Concentration of antibody titres to a) Hib, b) pertussis, c) tetanus and d) HBV measured at 14, 24, and 52 weeks of age in HIV-exposed infants (dark grey dots) vs. HIV-unexposed infant (light grey dots), regardless of timing of BCG vaccination. Shaded area indicates "protective" antibody concentrations. Bar and error bars represent geometric mean concentration with 95% confidence interval.

Table 2. Proportions of infants with "protective" antibody levels to Haemophilus influenza b, whole cell pertussis, tetanus toxoid and hepatitis B vaccines comparing HIV-exposed and –unexposed infants, regardless of timing of BCG vaccination (summary data).

Table 3. Multiple linear regression for predictors of "protective" antibody titres to Haemophilus influenzae b conjugate vaccine and whole cell B. pertussis at 14 weeks of age (N=165).

Supplementary Table 1. Proportion of infants with "protective" antibody levels to Haemophilus influenza b, whole cell pertussis, tetanus toxoid and hepatitis B vaccines at 14, 24 and 52 weeks of age in infants receiving BCG at birth vs. infants receiving delayed BCG vaccination at 14 weeks of age.

Supplementary Table 2. Antibody concentrations Haemophilus influenza b (HIB), whole cell pertussis, tetanus toxoid (TT), and hepatitis B vaccines (HBV) at 14, 24 and 52 weeks (N=165) regardless of HIV exposure or timing of BCG vaccination (summary analysis).

Supplementary Figure 1. Antibody titres in HIV-exposed infants to a) Hib, b) pertussis, c) tetanus and d) HBV measured at 14, 24, and 52 weeks of age in infants who received BCG vaccination at birth (black dots) vs. infants who received delayed BCG vaccination at 14 weeks of age (white circles). Shaded area indicates "protective" antibody concentrations. Bar and error bars represent geometric mean concentration (GMC) with 95% confidence interval.

**Table 1**

| | BCG at birth (N=106) | | | Delayed BCG at 14 weeks (N=74) | | | Comparison of birth vs. delayed arms |
|---|---|---|---|---|---|---|---|
| | HIV-exposed (N=67, 63.2%)) | HIV-unexposed (N=39, 36.8%) | p value | HIV-exposed (N=46, 62.2%) | HIV-unexposed (N=28, 37.8%) | p value | p value |
| Female sex (%) | 31 (46.2%) | 18 (46.2%) | 0.99 | 21 (45.6%) | 13 (46.4%) | 0.95 | 0.97 |
| Mean birth weight, grams (standard error) | 3250 (60) | 3240 (120) | 0.95 | 3170 (60) | 3200 (80) | 0.73 | 0.42 |
| Mean maternal CD4+ T cell count (standard error) | 355 (28) | N/A | | 386 (28) | N/A | | 0.47 |
| Maternal highly active antiretroviral therapy (%) | 34 (50.7%) | N/A | | 31 (67.4%) | N/A | | 0.077 |

**Table 2**

| | ***Haemophilus influenzae* B** **Frequency (%)** **Protective levels (>1 mg/ml)** | | | **Pertussis** **Frequency (%)** **Positive ("protected") (>30 FDA-U/ml)** | | | **Tetanus** **Frequency (%)** **"Sufficient or long-term immunity" (>0.1 IU/ml)** | | | **Hepatitis B** **Frequency (%)** **Positive ("protected") (≥10 mIU/ml)** | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | HIV-exposed N (%) | HIV-unexposed N (%) | Odds ratio (95% CI) | HIV-exposed N (%) | HIV-unexposed N (%) | Odds ratio (95% CI) | HIV-exposed N (%) | HIV-unexposed N (%) | Odds ratio (95% CI) | HIV-exposed N (%) | HIV-unexposed N (%) | Odds ratio (95% CI) |
| Week 14 | 69/101 (68.3) | 27/58 (46.6) | 1.75 (1.17-2.63) | 47/101 (46.5) | 27/60 (44.8) | 1.05 (0.69-1.58) | 100/100 (100.0) | 58/58 (100.0) | N/A | 80/80 (100.0) | 50/51 (98.0) | N/A |
| Week 24 | 68/94 (72.3)) | 24/55 (43.6) | 2.09 (1.37-3.17) | 60/85 (70.6) | 25/53 (47.2) | 1.80 (1.19-2.72) | 86/86 (100.0) | 63/63 (100.0) | N/A | 93/93 (100.0) | 48/48 (100.0) | N/A |
| Week 52 | 21/54 (38.9) | 8/30 (26.7) | 1.45 (0.74-2.84) | 31/51 (60.4) | 18/30 (60.0) | 1.01 (0.56-1.81) | 52/53 (98.1) | 29/29 (100.0) | 0.08 (0.96-1.02) | 28/28 (100.0) | 20/20 (100.0) | N/A |

**Table 3**

| | ***Haemophilus influenzae b*** | | | **Pertussis** | | |
|---|---|---|---|---|---|---|
| | Coefficient | 95% Confidence Interval | p value | Coefficient | 95% Confidence Interval | p value |
| BCG vaccination given at birth | -0.20 | -0.45-0.04 | 0.102 | -0.14 | -0.34—0.05 | 0.151 |
| HIV exposure | 0.47 | 0.21-0.72 | **<0.001** | 0.16 | -0.04-0.36 | 0.121 |
| Birth weight (kg) | 0.18 | -0.06-0.41 | 0.149 | -0.02 | -0.21-0.17 | 0.839 |
| Male sex | 0.07 | -0.17-0.31 | 0.567 | 0.15 | 0.33-1.28 | 0.128 |

Figure 1

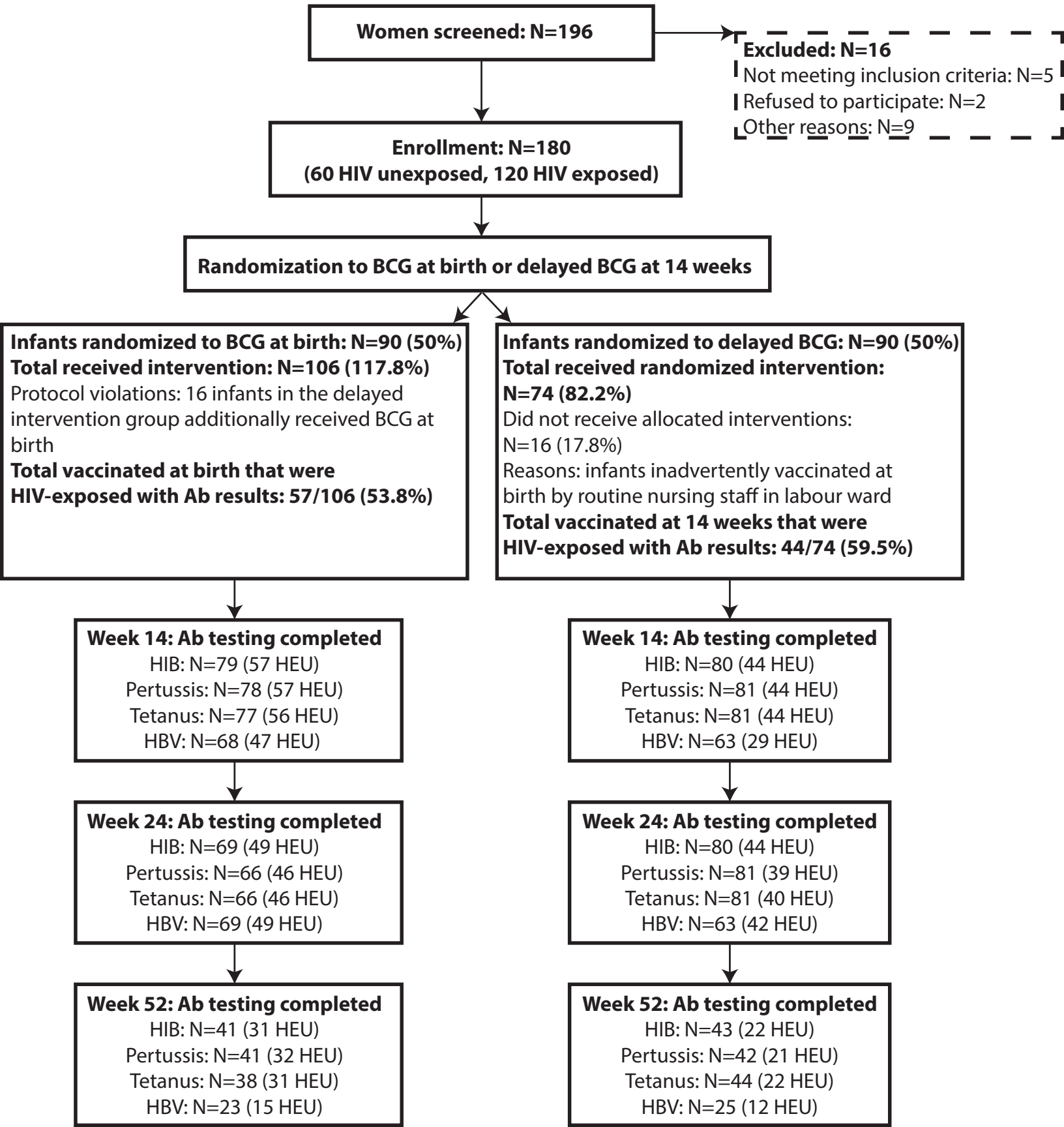

Women screened: N=196
Excluded: N=16
Not meeting inclusion criteria: N=5
Refused to participate: N=2
Other reasons: N=9
Enrollment: N=180
(60 HIV unexposed, 120 HIV exposed)
Randomization to BCG at birth or delayed BCG at 14 weeks
Infants randomized to BCG at birth: N=90 (50%)
Total received intervention: N=106 (117.8%)
Protocol violations: 16 infants in the delayed intervention group additionally received BCG at birth
Total vaccinated at birth that were HIV-exposed with Ab results: 57/106 (53.8%)
Infants randomized to delayed BCG: N=90 (50%)
Total received randomized intervention: N=74 (82.2%)
Did not receive allocated interventions: N=16 (17.8%)
Reasons: infants inadvertently vaccinated at birth by routine nursing staff in labour ward
Total vaccinated at 14 weeks that were HIV-exposed with Ab results: 44/74 (59.5%)
Week 14: Ab testing completed
HIB: N=79 (57 HEU)
Pertussis: N=78 (57 HEU)
Tetanus: N=77 (56 HEU)
HBV: N=68 (47 HEU)
Week 14: Ab testing completed
HIB: N=80 (44 HEU)
Pertussis: N=81 (44 HEU)
Tetanus: N=81 (44 HEU)
HBV: N=63 (29 HEU)
Week 24: Ab testing completed
HIB: N=69 (49 HEU)
Pertussis: N=66 (46 HEU)
Tetanus: N=66 (46 HEU)
HBV: N=69 (49 HEU)
Week 24: Ab testing completed
HIB: N=80 (44 HEU)
Pertussis: N=81 (39 HEU)
Tetanus: N=81 (40 HEU)
HBV: N=63 (42 HEU)
Week 52: Ab testing completed
HIB: N=41 (31 HEU)
Pertussis: N=41 (32 HEU)
Tetanus: N=38 (31 HEU)
HBV: N=23 (15 HEU)
Week 52: Ab testing completed
HIB: N=43 (22 HEU)
Pertussis: N=42 (21 HEU)
Tetanus: N=44 (22 HEU)
HBV: N=25 (12 HEU)

**Figure 2**

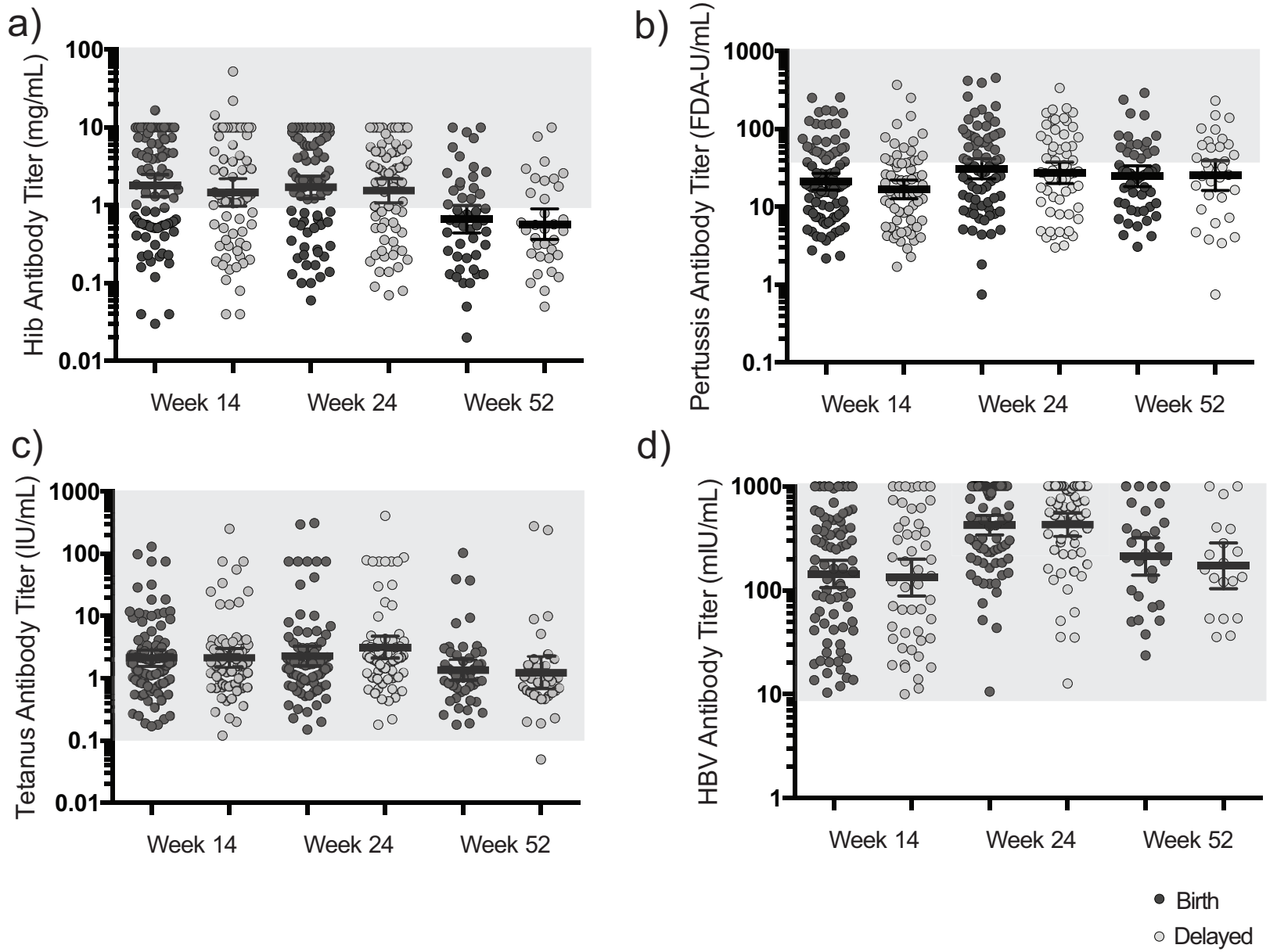
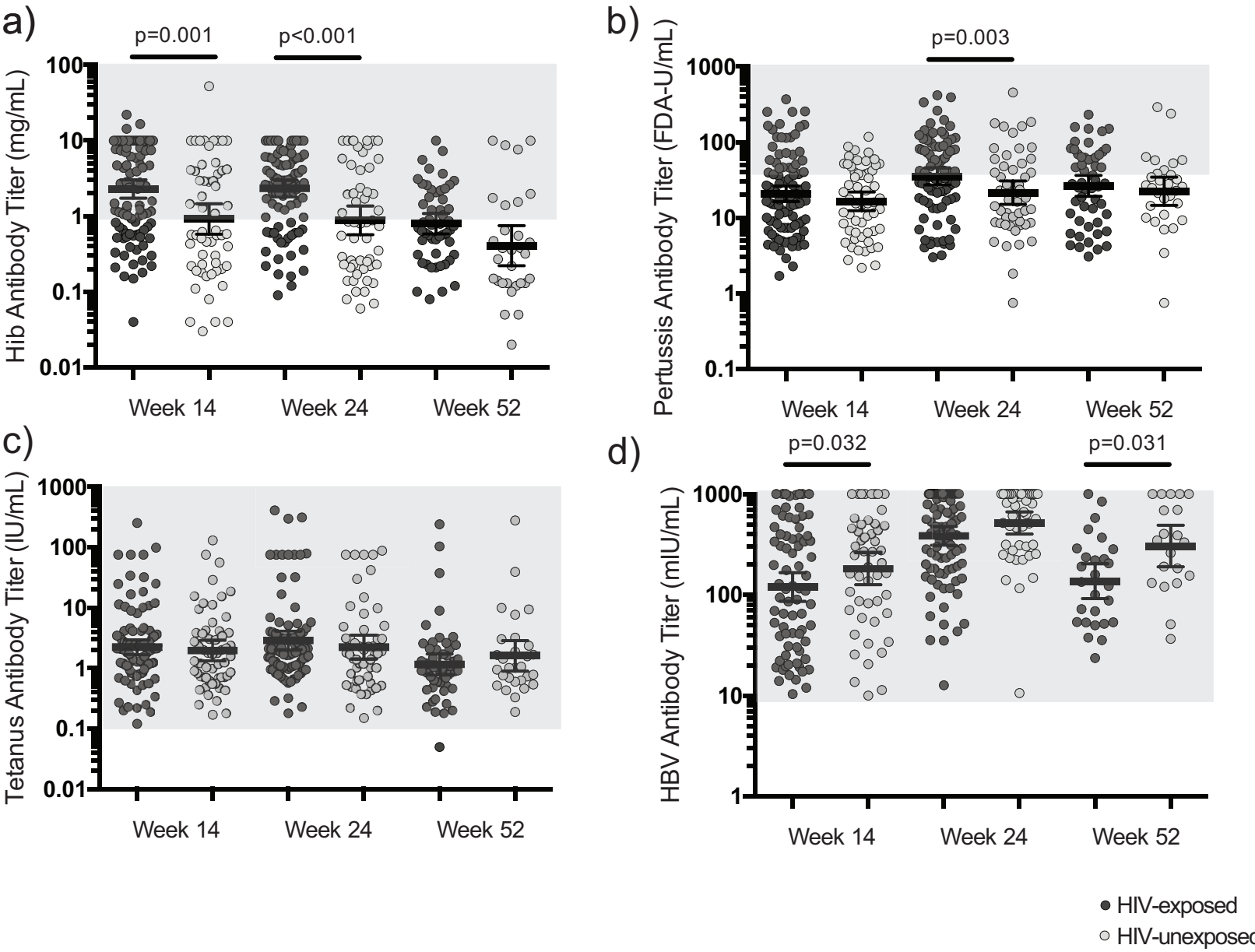

a)
Hib Antibody Titer (mg/mL)
100
10
1
0.1
0.01
Week 14
Week 24
Week 52
b)
Pertussis Antibody Titer (FDA-U/mL)
1000
100
10
1
0.1
Week 14
Week 24
Week 52
c)
Tetanus Antibody Titer (IU/mL)
1000
100
10
1
0.1
0.01
Week 14
Week 24
Week 52
d)
HBV Antibody Titer (mIU/mL)
1000
100
10
1
Week 14
Week 24
Week 52
Birth
Delayed

**Figure 3**


a)
p=0.001
p<0.001
Hib Antibody Titer (mg/mL)
100
10
1
0.1
0.01
Week 14
Week 24
Week 52
b)
p=0.003
Pertussis Antibody Titer (FDA-U/mL)
1000
100
10
1
0.1
Week 14
Week 24
Week 52
c)
Tetanus Antibody Titer (IU/mL)
1000
100
10
1
0.1
0.01
Week 14
Week 24
Week 52
d)
p=0.032
p=0.031
HBV Antibody Titer (mIU/mL)
1000
100
10
1
Week 14
Week 24
Week 52
HIV-exposed
HIV-unexposed

**Supplementary Figure 1**

**Click here to download Supplemental Files: SupplementaryFigure1.eps**

**Supplementary Table 1**

**Click here to download Supplemental Files: SupplementaryTable1.docx**

**Supplementary Table 2**

**Click here to download Supplemental Files: SupplementaryTable2.docx**