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# A multidisciplinary journey to approach public health challenges

Marta Nunes

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**Mémoire présenté en vue de l'obtention de  
L'HABILITATION À DIRIGER DES RECHERCHES**

***Marta Nunes***

**A multidisciplinary journey to approach public  
health challenges**

**Un parcours multidisciplinaire afin de relever les  
défis de la recherche en santé publique**

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## Abstract

The focus of my research has been on the epidemiology of vaccine preventable diseases. This included the evaluation of pneumococcal conjugate vaccines in a high HIV-burden setting, epidemiology of respiratory viral associated-pneumonia, and the impact of vaccination during pregnancy on the foetus and newborn. Included in my research portfolio are two pivotal randomized placebo-controlled trials of influenza vaccination during pregnancy and a third trial, using different vaccination regimens among HIV-infected women. These trials demonstrated the effect of vaccinating pregnant women on the prevention of influenza illness in the vaccinated mothers and their infants. The findings from the studies were pivotal in the Scientific Advisory Group of Experts (SAGE) formulating World Health Organization (WHO) recommendations for the prioritisation of influenza vaccination of pregnant women in low-middle income countries, and for the change in indication and labelling of influenza vaccines in Europe to include pregnancy. I conducted large epidemiological studies on the burden of COVID-19 among pregnant women, hospitalized adults and healthcare workers. Furthermore, in a prospective hospital-based surveillance I investigated the burden of Influenza, Respiratory Syncytial Virus (RSV), and Pertussis infections in children. With the rollout of COVID-19 vaccines, I was involved in vaccine effectiveness evaluation studies in South Africa, Eswatini, Botswana and Mozambique.

The COVID-19 pandemic has raised the attention on the substantial health burden respiratory viruses can cause to humans worldwide, emphasizing more than ever the importance to continue producing reliable burden of disease estimates. For the next steps of my research I will develop projects that investigate respiratory viruses' seasonality and viral evolution, as this is extremely relevant and will allow the identification of the populations most at-risk. I will perform post-registration evaluation for new vaccines and other preventive measures aiming to establish the optimal use of vaccines in France and globally. I will leverage on data, infrastructures, and expertise available from several existing partners at local, national, and global levels to achieve the following objectives:

1. Understand the burden and epidemiology of respiratory viruses that cause disease in very young and/or older age groups, which could lead to the development of improved or new vaccines.

Justification: traditionally the burden of respiratory infections displays a U shape curve relative to age groups, protecting these groups will be most effective to prevent illness and transmission. Additionally, robust science and evidence-based information are needed to support policy decisions and industrial investment in new vaccines.

2. Monitor the genetic evolution of respiratory viruses and associated mutations.

Justification: information on viruses' genetic evolution dynamics will guide research and development for future vaccine targets and formulations. Moreover, viral genetic information can be used by sentinel systems to identify new viral threats (e.g., changes in phenotype representation of viruses that could lead to more severe diseases) or interpret why certain vaccines are not performing well.

3. Evaluate the real-life performance of vaccines against different outcomes.

Justification: real-world evidence can be used for post-registration evaluation.

I will integrate and analyse large longitudinal data routinely collected by the Hospices Civils de Lyon (HCL). For the projects focused on French national information, I will use French National Health Insurance (SNDS) data. I will also use the Global Initiative on Sharing All Influenza Data (GISAID) platform to obtain genetic sequences and related clinical and epidemiological data from different geographical areas.

This project is a multi-disciplinary applied research program that will use information from surveillance platforms, clinical records, viral phenotypic and genomic data to better understand the circulation of respiratory viruses, their clinical impact and inform future changes in epidemiology that might occur.

## Résumé

Au cours de ma carrière, mes recherches se sont concentrées sur l'épidémiologie des maladies qui peuvent être prévenues par la vaccination. J'ai notamment évalué les vaccins conjugués antipneumococciques dans un contexte de forte prévalence du VIH, l'épidémiologie de la pneumonie virale associée aux voies respiratoires et l'impact de la vaccination pendant la grossesse sur le fœtus et le nouveau-né. Mon expérience en recherche comprend deux essais randomisés contrôlés par placebo sur la vaccination antigrippale pendant la grossesse et un troisième essai utilisant différents schémas de vaccination chez les femmes infectées par le VIH. Ces essais ont démontré l'effet de la vaccination des femmes enceintes sur la prévention de la grippe chez les mères vaccinées et leurs nourrissons. Les résultats de ces études ont joué un rôle essentiel dans la formulation par le groupe consultatif scientifique d'experts (SAGE) des recommandations de l'Organisation mondiale de la santé (OMS) visant à donner la priorité à la vaccination antigrippale des femmes enceintes dans les pays à revenu faible et intermédiaire et à modifier l'indication et l'étiquetage des vaccins antigrippaux en Europe afin d'y inclure la grossesse. J'ai mené de vastes études épidémiologiques sur la charge de COVID-19 chez les femmes enceintes, les adultes hospitalisés et le personnel de santé. Par ailleurs, dans le cadre d'une surveillance prospective en milieu hospitalier, j'ai étudié la charge des infections par la grippe, le virus respiratoire syncytial (VRS) et la coqueluche chez les enfants. Avec le déploiement des vaccins COVID-19, j'ai participé à des études d'évaluation de l'efficacité des vaccins en Afrique du Sud, en Eswatini, au Botswana et au Mozambique.

La pandémie de COVID-19 a attiré l'attention sur la charge de morbidité considérable que les virus respiratoires peuvent faire peser sur les personnes dans le monde entier, soulignant plus que jamais l'importance de continuer à produire des estimations fiables de la charge de morbidité. Pour les prochaines étapes de mes recherches, je développerai des projets visant à étudier la saisonnalité et l'évolution virale des virus respiratoires, ce qui permettra d'identifier les populations les plus à risque. Je réaliserai des évaluations après autorisation de mise sur le marché pour les nouveaux vaccins et autres mesures préventives afin d'établir l'utilisation optimale des vaccins en France et dans le monde. Je m'appuierai sur les données, les infrastructures et l'expertise disponibles auprès de plusieurs partenaires existants aux niveaux local, national et mondial pour atteindre les objectifs suivants :

1. Comprendre la charge et l'épidémiologie des virus respiratoires qui provoquent des maladies chez les très jeunes et/ou les personnes âgées, ce qui pourrait conduire au développement de vaccins nouveaux ou améliorés.

Traditionnellement, la charge des infections respiratoires présente une courbe en forme de U par rapport aux groupes d'âge, et la protection de ces groupes sera la plus efficace pour prévenir la maladie et la transmission. De plus, des données scientifiques solides et des informations fondées sur des preuves sont nécessaires pour étayer les décisions politiques et les investissements industriels vers de nouveaux vaccins.

2. Surveiller l'évolution génétique des virus respiratoires et les mutations associées.

Les informations sur la dynamique de l'évolution génétique des virus orienteront la recherche et le développement de futures cibles et formulations de vaccins. De plus, les informations génétiques virales peuvent être utilisées par les systèmes sentinelles pour identifier de nouvelles pistes virales (par exemple, des changements dans la représentation phénotypique des virus qui pourraient conduire à des maladies plus graves) ou pour interpréter les raisons pour lesquelles certains vaccins ne donnent pas de bons résultats.

3. Évaluer la performance des vaccins dans la vie réelle par rapport à différents résultats.

Les données de vie réelle peuvent être utilisées pour l'évaluation après la mise sur le marché des vaccins.

J'intégrerai et j'analyserai les données longitudinales importantes collectées régulièrement par les Hospices Civils de Lyon (HCL). Pour les projets axés sur les informations nationales françaises, j'utiliserai les données de l'Assurance maladie française (SNDS). J'utiliserai également la plateforme GISAID (Global Initiative on Sharing All Influenza Data) pour obtenir des séquences génétiques et des données cliniques et épidémiologiques connexes provenant de différentes zones géographiques.

Ce projet est un programme de recherche appliquée multidisciplinaire qui utilisera des informations provenant de plateformes de surveillance, de dossiers cliniques, de données phénotypiques et génomiques virales afin de mieux comprendre la circulation des virus respiratoires, leur impact clinique et d'informer sur les changements épidémiologiques qui pourraient survenir à l'avenir.



# Curriculum Vitae

Name: Marta Sofia Coelho Nunes

Date of Birth: May 18, 1974

Nationality: Portugal

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## Education

October 1999 – June 2003: PhD in Biomedical Sciences with distinction (score 20/20); Department of Neurology & Neuroscience, Weill Medical College of Cornell University, New York, NY, USA & University of Lisbon, Lisbon, Portugal. PhD thesis title: Identification and isolation of multipotential neural progenitor cells from adult human subcortical white matter. Supervisor: Steven Goldman (New York, USA). National supervisor: João Lobo Antunes (Lisbon, Portugal).

October 1998 – August 1999: Graduate courses and laboratory rotations for the Gulbenkian PhD Program in Biology and Medicine; Instituto Gulbenkian de Ciência, Oeiras, Portugal.

October 1996 – June 1997: Final research project for the degree in Biochemistry in the field of enzymology and molecular biology; Biochemistry Department, Bergen University, Bergen, Norway. Supervisor: Abraham K. Abraham (Bergen, Norway). National supervisor: Carlos Faro (Coimbra, Portugal).

October 1992 – June 1997: Degree in Biochemistry; Faculdade de Ciências e Tecnologia, Universidade de Coimbra, Coimbra, Portugal.

## Professional experience

September 2022 – Present: Center of Excellence for Respiratory Pathogens, Hospices Civils de Lyon, Lyon, France. Director.

September 2022 – Present: Université Claude Bernard Lyon 1, Lyon, France. Professeur à l'université.

June 2022 – Present: Faculty of Health Sciences, University of the Witwatersrand, South Africa. Research Professor.

June 2021 – December 2022: Department of Science and Technology (DST)/National Research Foundation (NRF), South African Research Chair: Vaccine Preventable Diseases. Chair.

March 2018 – May 2022: School of Pathology, Faculty of Health Sciences, University of the Witwatersrand, South Africa. Reader, Associate Professor.

March 2014 – July 2022: Vaccine Preventable Diseases Unit and Vaccines & Infectious Diseases Analytics (VIDA) Research Unit, University of the Witwatersrand, South Africa. Senior Researcher.

March 2009 – February 2014: Vaccine Preventable Diseases Unit and Respiratory and Meningeal Pathogens Research Unit (RMPRU), University of the Witwatersrand, South Africa. Post-doctoral Fellow. Area of research: Vaccine preventable diseases and maternal immunization. Host: Shabir Madhi.

March 2008 – February 2009: Theoretical Epidemiology Group, Instituto Gulbenkian de Ciência, Oeiras, Portugal. Research Scientist. Area of research: Malaria and Tuberculosis epidemiology.

October 2003 – February 2008: Parasitologie, Institut Pasteur, Paris, France. Post-doctoral Fellow. Area of research: Antigenic variation in Plasmodium Falciparum and pregnancy malaria vaccines. Host: Artur Scherf.

September 1997 – September 1998: Instituto de Tecnologia Química e Biológica, Universidade NOVA de Lisboa (ITQB), New University of Lisbon, Oeiras, Portugal. Junior Research Fellow. Area of research: Characterization of metabolic fluxes in lactic bacteria with industrial interest. Host: Helena Santos.

## Indexes

h-Index GoogleScholar: 44

i10-Index GoogleScholar: 100

South African NRF rated scientist in the B category at level B2.

## Peer-reviewed publications

1. Caini S, Meijer A, **Nunes MC**, Henaff L, Zounon M, Boudewijns B, Del Riccio M, Paget J. Probable extinction of influenza B/Yamagata and its public health implications: a systematic literature review and assessment of global surveillance databases. *Lancet Microbe*. 2024 May 7:S2666-5247(24)00066-1.
2. Horvat C, Chauvel C, Casalegno JS, Benchaib M, Ploin D, **Nunes MC**; VRS Study Group in Lyon. RSV Severe Infection Risk Stratification in a French 5-Year Birth Cohort Using Machine-learning. *Pediatr Infect Dis J*. 2024 May 7.
3. Motsoeneng BM, Dhar N, **Nunes MC**, Krammer F, Madhi SA, Moore PL, Richardson SI. Hemagglutinin stalk-specific Fc-mediated functions are associated with protection against influenza-illness after seasonal influenza vaccination in pregnant women. *J Infect Dis*. 2024 May 14:jiae241.
4. Ulrichs T, Rolland M, Wu J, **Nunes MC**, El Guerche-Séblain C, Chit A. Changing epidemiology of COVID-19: potential future impact on vaccines and vaccination strategies. *Expert Rev Vaccines*. 2024 Jan-Dec;23(1):510-522.
5. **Nunes MC**, Thommes E, Fröhlich H, Flahault A, Arino J, Baguelin M, et al. Redefining pandemic preparedness: Multidisciplinary insights from the CERP modelling workshop in infectious diseases, workshop report. *Infect Dis Model*. 2024 Jun;9(2):501–18.
6. Johnson M, Lazarus SK, Bennett AE, Tovar-Salazar A, Robertson CE, Kofonow JM, Li S, McCollister B, **Nunes MC**, Madhi SA, Frank DN, Weinberg A. Gut Microbiota and Other Factors Associated With Increased Regulatory T Cells in Hiv-exposed Uninfected Infants. *Res Sq [Preprint]*. 2024 Feb 2:rs.3.rs-3909424.
7. Langedijk AC, Vrancken B, Lebbink RJ, Wilkins D, Kelly EJ, Baraldi E, Mascareñas de Los Santos AH, Danilenko DM, Choi EH, Palomino MA, Chi H, Keller C, Cohen R, Papenburg J, Pernica J, Greenough A, Richmond P, Martínón-Torres F, Heikkinen T, Stein RT, Hosoya M, **Nunes MC**, Verwey C, Evers A, Kragten-Tabatabaie L, Suchard MA, Kosakovsky Pond SL, Poletto C, Colizza V, Lemey P, Bont LJ; INFORM-RSV Study Group. The genomic evolutionary dynamics and global circulation patterns of respiratory syncytial virus. *Nat Commun*. 2024 Apr 10;15(1):3083.
8. Schwalbe N, **Nunes MC**, Cutland C, Wahl B, Reidpath D. Assessing New York City's COVID-19 Vaccine Rollout Strategy: A Case for Risk-Informed Distribution. *J Urban Health*. 2024 Apr 5.
9. Mbotwe-Sibanda S, Kwatra G, Madhi SA, **Nunes MC**. Post-acute sequelae of SARS-CoV-2 infection in health care workers from South Africa. *Oxf Open Immunol*. 2024 Mar 14;5(1):iqae001.
10. Mutevedzi PC, Lokotfwako V, Kwatra G, Maphalala G, Baillie V, Dlamini L, Dlamini S, Mhlanga F, Dlamini T, Nhlabatsi N, **Nunes MC**, Zwane S, Madhi SA. Prevalence and Levels of Anti-SARS-CoV-2 Antibodies in the Eswatini Population and Subsequent Severity of the Fourth COVID-19 Epidemic Wave. *COVID*. 2024 Feb 20;4(3):317–33.
11. Wang X, Li Y, Shi T, Bont LJ, Chu HY, Zar HJ, Wahi-Singh B, Ma Y, Cong B, Sharland E, Riley RD, Deng J, Figueras-Aloy J, Heikkinen T, Jones MH, Liese JG, Markić J, Mejias A, **Nunes MC**, Resch B, Satav A, Yeo KT, Simões EAF, Nair H; Respiratory Virus Global Epidemiology Network; RESCEU investigators. Global disease burden of and risk factors for acute lower respiratory infections caused by respiratory syncytial virus in preterm

infants and young children in 2019: a systematic review and meta-analysis of aggregated and individual participant data. *Lancet*. 2024 Mar 30;403(10433):1241-1253.

12. Elias C, **Nunes MC**, Saadatian-Elahi M. Epidemiology of community-acquired pneumonia caused by *Streptococcus pneumoniae* in older adults: a narrative review. *Curr Opin Infect Dis*. 2024 Apr;37(2):144–53.

13. Tomasicchio M, Jaumdally S, Wilson L, Kotze A, Semple L, Meier S, Pooran A, Esmail A, Pillay K, Roberts R, Kriel R, Meldau R, Oelofse S, Mandviwala C, Burns J, Londt R, Davids M, van der Merwe C, Roomaney A, Kühn L, Perumal T, Scott AJ, Hale MJ, Baillie V, Mahtab S, Williamson C, Joseph R, Sigal A, Joubert I, Piercy J, Thomson D, Fredericks DL, Miller MG, **Nunes MC**, Madhi SA, Dheda K. SARS-CoV-2 Viral Replication Persists in the Human Lung for Several Weeks after Symptom Onset. *Am J Respir Crit Care Med*. 2024 Apr 1;209(7):840-851.

14. Moosa F, du Plessis M, Weigand MR, Peng Y, Mogale D, de Gouveia L, **Nunes MC**, Madhi SA, Zar HJ, Reubenson G, Ismail A, Tondella ML, Cohen C, Walaza S, von Gottberg A, Wolter N. Genomic characterization of *Bordetella pertussis* in South Africa, 2015-2019. *Microb Genom*. 2023;9(12):001162.

15. **Nunes MC**, Tambllyn A, Jose L, Ntsimane M, Lerotholi N, Machimana C, Taylor A, Laher F, Madhi SA. Immunogenicity of tetanus, diphtheria and acellular pertussis vaccination among pregnant women living with and without HIV. *AIDS*. 2023; 37(15):2305–10.

16. Olwagen CP, Downs SL, Izu A, Tharasimbi L, Van Der Merwe L, **Nunes MC**, Madhi SA. Bacterial nasopharyngeal colonisation in children in South Africa before and during the COVID-19 pandemic: an observational study. *Lancet Microbe*. 2024 Jan;5(1):e34-e42.

17. Olwagen CP, Jeché TR, Van Der Merwe L, **Nunes MC**, Madhi SA, Baillie VL. Nanofluidic qPCR unable to detect and serotype *Streptococcus pneumoniae* in urine samples of hospitalized South African patients with community-acquired pneumonia. *Sci Rep*. 2023;13(1):21332.

18. Andrew MK, Pott H, Staadegaard L, Paget J, Chaves SS, Ortiz JR, McCauley J, Bresee J, **Nunes MC**, Baumeister E, Raboni SM, Giamberardino HIG, McNeil SA, Gomez D, Zhang T, Vanhems P, Koul PA, Coulibaly D, Otieno NA, Dbaibo G, Almeida MLG, Laguna-Torres VA, Drăgănescu AC, Burtseva E, Sominina A, Danilenko D, Medić S, Diez-Domingo J, Lina B. Age Differences in Comorbidities, Presenting Symptoms, and Outcomes of Influenza Illness Requiring Hospitalization: A Worldwide Perspective From the Global Influenza Hospital Surveillance Network. *Open Forum Infect Dis*. 2023 May 7;10(6):ofad244.

19. Wilson L, Chang JW, Meier S, Ganief T, Ganief N, Oelofse S, Baillie V, **Nunes MC**, Madhi SA, Blackburn J, Dheda K. Proteomic Profiling of Urine From Hospitalized Patients With Severe Pneumonia due to SARS-CoV-2 vs Other Causes: A Preliminary Report. *Open Forum Infect Dis*. 2023 Aug 31;10(10):ofad451.

20. Kaplonek P, Cizmeci D, Kwatra G, Izu A, Lee JS, Bertera HL, Fischinger S, Mann C, Amanat F, Wang W, Koen AL, Fairlie L, Cutland CL, Ahmed K, Dheda K, Barnabas SL, Bhorat QE, Briner C, Krammer F, Saphire EO, Gilbert SC, Lambe T, Pollard AJ, **Nunes M**, Wuhrer M, Lauffenburger DA, Madhi SA, Alter G. ChAdOx1 nCoV-19 (AZD1222) vaccine-induced Fc receptor binding tracks with differential susceptibility to COVID-19. *Nat Immunol*. 2023;24(7):1161-1172.

21. Dunne EM, **Nunes MC**, Slack MPE, Theilacker C, Gessner BD. Effects of pneumococcal conjugate vaccines on reducing the risk of respiratory disease associated with coronavirus infection. *Pneumonia*. 2023;15(1):10.
22. Saadatian-Elahi M, Henaff L, Elias C, **Nunes MC**, Hot A, Martin-Gaujard G, Escuret V, Amour S, Vanhems P. Patient influenza vaccination reduces the risk of hospital-acquired influenza: An incident test negative-case control study in Lyon university hospital, France (2004-2020). *Vaccine*. 2023;41(30):4341-4346.
23. Cohen LE, Hansen CL, Andrew MK, McNeil SA, Vanhems P, Kyncl J, Díez Domingo J, Zhang T, Dbaibo G, Laguna-Torres VA, Draganescu A, Baumeister E, Gomez D, Raboni SM, Giamberardino HIG, **Nunes MC**, Burtseva E, Sominina A, Medić S, Coulibaly D, Salah AB, Otieno NA, Koul PA, Unal S, Tanriover MD, Mazur M, Bresee J, Viboud C, Chaves SS. Predictors of Severity of Influenza-Related Hospitalizations: Results From the Global Influenza Hospital Surveillance Network (GIHSN). *J Infect Dis*. 2024 Apr 12;229(4):999-1009.
24. Downs SL, Olwage CP, Van der Merwe L, Nzenze S, **Nunes MC**, Madhi SA. *Streptococcus pneumoniae* and other bacterial nasopharyngeal colonisation eight years post-introduction of 13-valent pneumococcal conjugate vaccine in South African children. *Int J Infect Dis*. 2023;134:45-52.
25. Verwey C, Ramocha L, Laubscher M, Baillie V, **Nunes M**, Gray D, Hantos Z, Dangor Z, Madhi S. Pulmonary sequelae in 2-year-old children after hospitalisation for respiratory syncytial virus lower respiratory tract infection during infancy: an observational study. *BMJ Open Respir Res*. 2023 May;10(1):e001618.
26. Izu A, **Nunes MC**, Solomon F, Baillie V, Serafin N, Verwey C, Moore DP, Laubscher M, Ncube M, Olwage C, Dangor Z, Madhi SA. All-cause and pathogen-specific lower respiratory tract infection hospital admissions in children younger than 5 years during the COVID-19 pandemic (2020-22) compared with the pre-pandemic period (2015-19) in South Africa: an observational study. *Lancet Infect Dis*. 2023 Sep;23(9):1031-1041.
27. Hooli S, King C, McCollum ED, Colbourn T, Lufesi N, Mwansambo C, Gregory CJ, Thamthitawat S, Cutland C, Madhi SA, **Nunes MC**, Gessner BD, et al. In-Hospital Mortality Risk Stratification in Children Under 5 Years Old with Pneumonia with or without Pulse Oximetry: A Secondary Analysis of the Pneumonia REsearch Partnership to Assess WHO REcommendations (PREPARE) Dataset. *Int J Infect Dis*. 2023 Apr;129:240-250.
28. Downs SL, Madhi SA, Van der Merwe L, **Nunes MC**, Olwage CP. Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify of 15 bacterial species and 92 *Streptococcus pneumoniae* serotypes. *Sci Rep*. 2023;13(1):4588.
29. Wilkins D, Langedijk AC, Lebbink RJ, Morehouse C, Abram ME, Ahani B, Aksyuk AA, Baraldi E, Brady T, Chen AT, Chi H, Choi EH, Cohen R, Danilenko DM, Gopalakrishnan V, Greenough A, Heikkinen T, Hosoya M, Keller C, Kelly EJ, Kragten-Tabatabaie L, Martínón-Torres F, de Los Santos AHM, **Nunes MC**, Palomino MA, Papenburg J, Pernica JM, Richmond P, Stein RT, Tuffy KM, Verwey C, Esser MT, Tabor DE, Bont LJ; INFORM-RSV Study Group. Nirsevimab binding-site conservation in respiratory syncytial virus fusion glycoprotein worldwide between 1956 and 2021: an analysis of observational study sequencing data. *Lancet Infect Dis*. 2023 Jul;23(7):856-866.
30. Smith ER, Oakley E, Grandner GW, Rukundo G, Farooq F, Ferguson K, Baumann S, Waldorf KA, Afshar Y, Ahlberg M, Ahmadzia H, Akelo V, Aldrovandi G, Bevilacqua E, Bracero N, Brandt JS, Broutet N, Carrillo J, Conry J, Cosmi E, Crispi F, Crovatto F, Gil MDM, Delgado-Lopez C, Divakar H, Driscoll AJ, Favre G, Buhigas IF, Flaherman V, Gale C,

Godwin CL, Gottlieb S, Gratacos E, He S, Hernandez O, Jones S, Joshi S, Kalafat E, Khagayi S, Knight M, Kotloff K, Lanzone A, Longo VL, Le Doare K, Lees C, Litman E, Lokken EM, Madhi SA, Magee LA, Martinez-Portilla RJ, Metz TD, Miller ES, Money D, Mounghmaithong S, Mullins E, Nachega JB, **Nunes MC**, et al. Adverse maternal, fetal, and newborn outcomes among pregnant women with SARS-CoV-2 infection: an individual participant data meta-analysis. *BMJ Glob Health*. 2023;8(1):e009495.

31. McMahon WC, Kwatra G, Izu A, Koen AL, Greffrath J, Fairlie L, Patel F, Mukendi CK, Mbele NJ, Lala R, Burgers W A., **Nunes MC**, Cutland CL, Gilbert SC, Lambe T, Pollard AJ, Madhi SA. T-cell responses induced by ChAdOx1 nCoV-19 (AZD1222) vaccine to wild-type severe acute respiratory syndrome coronavirus 2 among people with and without HIV in South Africa. *AIDS*. 2023 Jan 1;37(1):105-112.

32. Ramocha LM, Dorfman JR, Groome M, Baillie V, Verwey C, Laubscher M, **Nunes MC**, Madhi SA. Human Metapneumovirus-Associated Hospitalization in HIV-1 Exposed Uninfected and HIV-1 Uninfected Children Less Than 5 Years in South Africa. *J Pediatric Infect Dis Soc*. 2023 Feb 9;12(1):53-55.

33. Mazur NI, Terstappen J, Baral R, Bardaji A, Beutels P, Buchholz UJ, Cohen C, Crowe JE Jr, Cutland CL, Eckert L, Feikin D, Fitzpatrick T, Fong Y, Graham BS, Heikkinen T, Higgins D, Hirve S, Klugman KP, Kragten-Tabatabaie L, Lemey P, Libster R, Lowensteyn Y, Mejjias A, Munoz FM, Munywoki PK, Mwananyanda L, Nair H, **Nunes MC**, Ramilo O, Richmond P, Ruckwardt TJ, Sande C, Srikantiah P, Thacker N, Waldstein KA, Weinberger D, Wildenbeest J, Wiseman D, Zar HJ, Zambon M, Bont L. Respiratory syncytial virus prevention within reach: the vaccine and monoclonal antibody landscape. *Lancet Infect Dis*. 2023 Jan;23(1):e2-e21.

34. Smith ER, Oakley E, Grandner GW, Rukundo G, Farooq F, Ferguson K, Baumann S, Waldorf KA, Afshar Y, Ahlberg M, Ahmadzia H, Akelo V, Aldrovandi G, Bevilacqua E, Bracero N, Brandt JS, Broutet N, Carrillo J, Conry J, Cosmi E, Crispi F, Croveto F, Gil MDM, Delgado-Lopez C, Divakar H, Driscoll AJ, Favre G, Buhigas IF, Flaherman V, Gale C, Godwin CL, Gottlieb S, Gratacos E, He S, Hernandez O, Jones S, Joshi S, Kalafat E, Khagayi S, Knight M, Kotloff K, Lanzone A, Longo VL, Le Doare K, Lees C, Litman E, Lokken EM, Madhi SA, Magee LA, Martinez-Portilla RJ, Metz TD, Miller ES, Money D, Mounghmaithong S, Mullins E, Nachega JB, **Nunes MC**, et al. Clinical risk factors of adverse outcomes among women with COVID-19 in the pregnancy and postpartum period: A sequential, prospective meta-analysis. *Am J Obstet Gynecol*. 2023;228(2):161-177.

35. Martin H, Falconer J, Addo-Yobo E, Aneja S, Arroyo LM, Asghar R, Awasthi S, Banajeh S, Bari A, Basnet S, Bavdekar A, Bhandari N, Bhatnagar S, Bhutta ZA, Brooks A, Chadha M, Chisaka N, Chou M, Clara AW, Colbourn T, Cutland C, D'Acremont V, Echavarría M, Gentile A, Gessner B, Gregory CJ, Hazir T, Hibberd PL, Hirve S, Hooli S, Iqbal I, Jeena P, Kartasasmita CB, King C, Libster R, Lodha R, Lozano JM, Lucero M, Lufesi N, MacLeod WB, Madhi SA, Mathew JL, Maulen-Radovan I, McCollum ED, Mino G, Mwansambo C, Neuman MI, Nguyen NTV, **Nunes MC**, et al. Assembling a global database of child pneumonia studies to inform WHO pneumonia management algorithm: Methodology and applications. *J Glob Health*. 2022;12:04075.

36. **Nunes MC**, Walaza S, Meiring S, Zar HJ, Reubenson G, McMorrow M, Tempia S, Rossi L, Itzikowitz R, Bishop K, Mathunjwa A, Wise A, Treurnicht FK, Hellferscee O, Laubscher M, Serafin N, Cutland CL, Madhi SA, Cohen C. Effectiveness of Influenza Vaccination of Pregnant Women for Prevention of Maternal and Early Infant Influenza-Associated Hospitalizations in South Africa: A Prospective Test-Negative Study. *Open Forum Infect Dis*. 2022 Oct 19;9(11):ofac552.

37. Schwalbe N, Hanbali L, **Nunes MC**, Lehtimaki S. Use of financial incentives to increase adult vaccination coverage: A narrative review of lessons learned from COVID-19 and other adult vaccination efforts. *Vaccine X*. 2022;12:100225.
38. May AK, Seymour H, Etheredge H, Maher H, **Nunes MC**, Madhi SA, Sokhela SM, Venter WDF, Martinson N, Nabeemeeah F, Cohen C, Moyes J, Walaza S, Tempia S, Kleynhans J, von Gottberg A, Nel J, Dawood H, Variava E, Tollman S, Kahn K, Herbst K, Wong EB, Tiemessen CT, van Blydenstein A, Murray L, Venter M, Fabian J, Ramsay M. Coronavirus Host Genomics Study: South Africa (COVIGen-SA). *Glob Health Epidemiol Genom*. 2022 Oct 6;2022:7405349.
39. Carbonell-Estrany X, Simões EAF, Bont LJ, Gentile A, Homaira N, Scotta MC, Stein RT, Torres JP, Sheikh J, Broor S, Khuri-Bulos N, Nokes DJ, Munywoki PK, Bassat Q, Sharma AK, Basnet S, Garba M, De Jesus-Cornejo J, Lupisan SP, **Nunes MC**, et al. Identifying the research, advocacy, policy and implementation needs for the prevention and management of respiratory syncytial virus lower respiratory tract infection in low- and middle-income countries. *Front Pediatr*. 2022;10:1033125.
40. Rees CA, Colbourn T, Hooli S, King C, Lufesi N, McCollum ED, Mwansambo C, Cutland C, Madhi SA, **Nunes M**, et al. Derivation and validation of a novel risk assessment tool to identify children aged 2-59 months at risk of hospitalised pneumonia-related mortality in 20 countries. *BMJ Glob Health*. 2022 Apr;7(4):e008143.
41. **Nunes MC**, Mbotwe-Sibanda S, Baillie VL, Kwatra G, Aguas R, Madhi SA. SARS-CoV-2 Omicron Symptomatic Infections in Previously Infected or Vaccinated South African Healthcare Workers. *Vaccines (Basel)*. 2022;10(3).
42. **Nunes MC**, Madhi SA. COVID-19 vaccines in pregnancy. *Trends Mol Med*. 2022;28(8):662-80.
43. **Nunes MC**, Jones S, Ditse Z, da Silva K, Serafin N, Strehlau R, Wise A, Burke M, Baba V, Baillie VL, Nzimande A, Jafta N, Adam M, Mlandu P, Melamu M, Phelp J, Feldman C, Adam Y, Madhi SA, Kwatra G. Maternal-foetal transfer of severe acute respiratory syndrome coronavirus 2 antibodies among women with and those without HIV infection. *AIDS*. 2022 Nov 1;36(13):1777-1782.
44. **Nunes MC**, Jones S, Strehlau R, Baba V, Ditse Z, da Silva K, Bothma L, Serafin N, Baillie VL, Kwatra G, Burke M, Wise A, Adam M, Mlandu P, Melamu M, Phelp J, Fraser W, Wright C, Zell E, Adam Y, Madhi SA. Active Intrapartum SARS-CoV-2 Infection and Pregnancy Outcomes. *Am J Perinatol*. 2022 Dec;39(S 01):S42-S48.
45. **Nunes MC**, Jones S, Strehlau R, Baba V, Ditse Z, da Silva K, Bothma L, Serafin N, Baillie VL, Kwatra G, Burke M, Wise A, Adam M, Mlandu P, Melamu M, Phelp J, Fraser W, Wright C, Zell E, Adam Y, Madhi SA. Antepartum SARS-CoV-2 infection and adverse birth outcomes in South African women. *J Glob Health*. 2022 Dec 3;12:05050.
46. **Nunes MC**, Hale MJ, Mahtab S, Mabena FC, Dlodlu N, Baillie VL, Thwala BN, Els T, du Plessis J, Laubscher M, Mckenzie S, Mtshali S, Menezes C, Serafin N, van Blydenstein S, Tsitsi M, Dulisse B, Madhi SA. Clinical characteristics and histopathology of COVID-19 related deaths in South African adults. *PLoS One*. 2022 Jan 20;17(1):e0262179.
47. Motsoeneng BM, Dhar N, **Nunes MC**, Krammer F, Madhi SA, Moore PL, Richardson SI. Influenza Vaccination Results in Differential Hemagglutinin Stalk-Specific Fc-Mediated Functions in Individuals Living With or Without HIV. *Front Immunol*. 2022 Apr 19;13:873191.

48. Kwatra G, **Nunes M**, Dhar N, Baillie V, Serafin N, Jones S, Madhi SA. Correlation of dried blood spots and plasma for quantification of Immunoglobulin (IgG) against Receptor binding domain and full length spike protein of SARS-CoV-2. *J Virol Methods*. 2022 Feb;300:114394.
49. Jackson CL, Frank DN, Robertson CE, Ir D, Kofonow JM, Montlha MP, Mutsaerts EAML, **Nunes MC**, Madhi SA, Ghosh D, Weinberg A. Evolution of the Gut Microbiome in HIV-Exposed Uninfected and Unexposed Infants during the First Year of Life. *mBio*. 2022 Oct 26;13(5):e0122922.
50. Dejnirattisai W, Huo J, Zhou D, Zahradnik J, Supasa P, Liu C, Duyvesteyn HME, Ginn HM, Mentzer AJ, Tuekprakhon A, Nutalai R, Wang B, Dijokaite A, Khan S, Avinoam O, Bahar M, Skelly D, Adele S, Johnson SA, Amin A, Ritter TG, Mason C, Dold C, Pan D, Assadi S, Bellass A, Omo-Dare N, Koeckerling D, Flaxman A, Jenkin D, Aley PK, Voysey M, Costa Clemens SA, Naveca FG, Nascimento V, Nascimento F, Fernandes da Costa C, Resende PC, Pauvolid-Correa A, Siqueira MM, Baillie V, Serafin N, Kwatra G, Da Silva K, Madhi SA, **Nunes MC**, Malik T, Openshaw PJM, Baillie JK, Semple MG, Townsend AR, Huang KA, Tan TK, Carroll MW, Klenerman P, Barnes E, Dunachie SJ, Constantinides B, Webster H, Crook D, Pollard AJ, Lambe T; OPTIC Consortium; ISARIC4C Consortium; Paterson NG, Williams MA, Hall DR, Fry EE, Mongkolsapaya J, Ren J, Schreiber G, Stuart DI, Screaton GR. SARS-CoV-2 Omicron-B.1.1.529 leads to widespread escape from neutralizing antibody responses. *Cell*. 2022 Feb 3;185(3):467-484.e15.
51. **Nunes MC**, Baillie VL, Kwatra G, Bhikha S, Verwey C, Menezes C, Cutland CL, Moore DP, Dangor Z, Adam Y, Mathivha R, Velaphi SC, Tsitsi M, Aguas R, Madhi SA; Bara HCW Study Group. Severe Acute Respiratory Syndrome Coronavirus 2 Infection Among Healthcare Workers in South Africa: A Longitudinal Cohort Study. *Clin Infect Dis*. 2021 Nov 16;73(10):1896-1900.
52. **Nunes MC**, Johnson MJ, Kwatra G, Weinberg A, Madhi SA. T-cell responses to SARS-CoV-2 in unexposed South African women. *Gates Open Res*. 2021;5:150.
53. Downs SL, Madhi SA, Van der Merwe L, **Nunes MC**, Olwagen CP. High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides. *Sci Rep*. 2021;11(1):23728.
54. Navarro Alonso JA, Bont LJ, Bozzola E, Herting E, Lega F, Mader S, **Nunes MC**, Ramilo O, Valiotis G, Olivier CW, Yates A, Faust SN. RSV: perspectives to strengthen the need for protection in all infants. *Emerg Themes Epidemiol*. 2021 Oct 21;18(1):15.
55. Rees CA, Hooli S, King C, McCollum ED, Colbourn T, Lufesi N, Mwansambo C, Lazzarini M, Madhi SA, Cutland C, **Nunes M**, Gessner BD, et al. External validation of the RISC, RISC-Malawi, and PERCH clinical prediction rules to identify risk of death in children hospitalized with pneumonia. *J Glob Health* 2021;11:04062.
56. Mabena FC, Baillie VL, Hale MJ, Thwala BN, Mthembu N, Els T, Serafin N, du Plessis J, Swart P, Velaphi SC, Petersen KL, Wadula J, Govender NP, Verwey C, Moore DP, Moosa FY, Nakwa FL, Maroane BV, Okudo G, Mabaso TM, Dangor Z, **Nunes MC**, Madhi SA. Clinical Characteristics and Histopathology of Coronavirus Disease 2019-Related Deaths in African Children. *Pediatr Infect Dis J*. 2021 Sep 1;40(9):e323-e332.
57. Liu C, Ginn HM, Dejnirattisai W, Supasa P, Wang B, Tuekprakhon A, Nutalai R, Zhou D, Mentzer AJ, Zhao Y, Duyvesteyn HME, Lopez-Camacho C, Slon-Campos J, Walter TS, Skelly D, Johnson SA, Ritter TG, Mason C, Costa Clemens SA, Gomes Naveca F,



- Nascimento V, Nascimento F, Fernandes da Costa C, Resende PC, Pauvolid-Correa A, Siqueira MM, Dold C, Temperton N, Dong T, Pollard AJ, Knight JC, Crook D, Lambe T, Clutterbuck E, Bibi S, Flaxman A, Bittaye M, Belij-Rammerstorfer S, Gilbert SC, Malik T, Carroll MW, Klenerman P, Barnes E, Dunachie SJ, Baillie V, Serafin N, Ditse Z, Da Silva K, Paterson NG, Williams MA, Hall DR, Madhi S, **Nunes MC**, Goulder P, Fry EE, Mongkolsapaya J, Ren J, Stuart DI, Sreaton GR. Reduced neutralization of SARS-CoV-2 B.1.617 by vaccine and convalescent serum. *Cell*. 2021 Aug 5;184(16):4220-4236.e13.
58. Löwensteyn YN, Nair H, **Nunes MC**, van Roessel I, Vernooij FS, Willemsen J, Bont LJ, Mazur NI; FLU GOLD study group. Estimated impact of maternal vaccination on global paediatric influenza-related in-hospital mortality: A retrospective case series. *EClinicalMedicine*. 2021 Jun 10;37:100945.
59. **Nunes MC**, Cutland CL, Klugman KP, Madhi SA. Pneumococcal Conjugate Vaccine Protection against Coronavirus-Associated Pneumonia Hospitalization in Children Living with and without HIV. *mBio* 2021;12(1).
60. Macias AE, McElhaney JE, Chaves SS, Nealon J, **Nunes MC**, Samson SI, Seet BT, Weinke T, Yu H. The disease burden of influenza beyond respiratory illness. *Vaccine*. 2021 Mar 15;39 Suppl 1:A6-A14.
61. Wang X, Li Y, Deloria-Knoll M, Madhi S A, Cohen C, Ali A, Basnet S, Bassat Q, Brooks WA, Chittaganpitch M, Echavarria M, Fasce RA, Goswami D, Hirve S, Homaira N, Howie SRC, Kotloff KL, Khuri-Bulos N, Krishnan A, Lucero MG, Lupisan S, Mira-Iglesias A, Moore DP, Moraleda C, **Nunes M**, Oshitani H, Owor BE, Polack FP, O'Brien KL, Rasmussen ZA, Rath BA, Salimi V, Scott JAG, Simões EAF, Strand TA, Thea DM, Treurnicht FK, Vaccari LC, Yoshida LM, Zar HJ, Campbell H, Nair H; Respiratory Virus Global Epidemiology Network. Global burden of acute lower respiratory infection associated with human metapneumovirus in children under 5 years in 2018: a systematic review and modelling study. *Lancet Glob Health*. 2021 Jan;9(1):e33-e43.
62. **Nunes MC**, Moreira M, Koen A, van Niekerk N, Jose L, Cutland CL, François N, Schoonbroodt S, Ruiz-Guiñazú J, Yarzabal JP, Borys D, Schuerman L, Madhi SA. Bacterial nasopharyngeal carriage following infant immunization with pneumococcal conjugate vaccines according to a 2+1 schedule in children in South Africa: an exploratory analysis of two clinical trials. *Expert Rev Vaccines*. 2020 Dec;19(12):1177-1189.
63. Amin AB, **Nunes MC**, Tapia MD, Madhi SA, Cutland CL, Wairagkar N, Omer SB; for BMGF Supported Maternal Influenza Immunization Trials Investigators Group. Immunogenicity of influenza vaccines administered to pregnant women in randomized clinical trials in Mali and South Africa. *Vaccine*. 2020 Sep 22;38(41):6478-6483.
64. Verwey C, **Nunes MC**, Dangor Z, Madhi SA. Pulmonary function sequelae after respiratory syncytial virus lower respiratory tract infection in children: A systematic review. *Pediatr Pulmonol*. 2020;55(7):1567-1583.
65. Langedijk AC, Lebbink RJ, Naaktgeboren C, Evers A, Viveen MC, Greenough A, Heikkinen T, Stein RT, Richmond P, Martínón-Torres F, **Nunes M**, Hosoya M, Keller C, Bauck M, Cohen R, Papenburg J, Pernica J, Hennis MP, Jin H, Tabor DE, Tovchigrechko A, Ruzin A, Abram ME, Wilkins D, Wildenbeest JG, Kragten-Tabatabaie L, Coenjaerts FEJ, Esser MT, Bont LJ. Global molecular diversity of RSV - the "INFORM RSV" study. *BMC Infect Dis*. 2020 Jun 26;20(1):450.
66. Lina B, Georges A, Burtseva E, **Nunes MC**, Andrew MK, McNeil SA, Ruiz-Palacios GM, Feng L, Kyncl J, Vanhems P, Ortiz JR, Paget J, Reiner RC; GIHSN 2017–2018 study

collaborators. Complicated hospitalization due to influenza: results from the Global Hospital Influenza Network for the 2017-2018 season. *BMC Infect Dis.* 2020 Jul 2;20(1):465.

67. Omer SB, Clark DR, Madhi SA, Tapia MD, **Nunes MC**, Cutland CL, Simões EAF, Aqil AR, Katz J, Tielsch JM, Steinhoff MC, Wairagkar N; BMGF Supported Maternal Influenza Immunization Trials Investigators Group. Efficacy, duration of protection, birth outcomes, and infant growth associated with influenza vaccination in pregnancy: a pooled analysis of three randomised controlled trials. *Lancet Respir Med.* 2020 Jun;8(6):597-608.

68. **Nunes MC**, Kim S, Zeldow B, Violari A, Dittmer S, Cassim H, Thomas T, van Niekerk N, Cotton MF, Mitchell C, Adrian P, Madhi SA; P1041 team. Streptococcus pneumoniae colonization in pneumococcal vaccine-naïve human immunodeficiency virus-exposed infected and -uninfected South African children. *Medicine (Baltimore).* 2020 Feb;99(9):e19353.

69. **Nunes MC**, Cutland CL, Moultrie A, Jones S, Ortiz JR, Neuzil KM, Klugman KP, Simões EAF, Weinberg A, Madhi SA; Maternal Flu Trial Team. Immunogenicity and safety of different dosing schedules of trivalent inactivated influenza vaccine in pregnant women with HIV: a randomised controlled trial. *Lancet HIV.* 2020 Feb;7(2):e91-e103.

70. Mutsaerts EAML, **Nunes MC**, Bhikha S, Ikulinda BT, Jose L, Koen A, Moultrie A, Grobbee DE, Klipstein-Grobusch K, Weinberg A, Madhi SA. Short-term immunogenicity and safety of hepatitis-A and varicella vaccines in HIV-exposed uninfected and HIV-unexposed South African children. *Vaccine.* 2020 May 8;38(22):3862-3868.

71. Madhi SA, Nzenze SA, **Nunes MC**, Chinyanganya L, Van Niekerk N, Kahn K, Twine R, De Gouveia L, Von Gottberg A, Shiri T. Residual colonization by vaccine serotypes in rural South Africa four years following initiation of pneumococcal conjugate vaccine immunization. *Expert Rev Vaccines.* 2020 Apr;19(4):383-393.

72. Liu H, Bin L, Tabor DE, Tovchigrechko A, Wilkins D, Jin H, Madhi SA, Soofie N, Esser MT, **Nunes MC**. Characterization of human respiratory syncytial virus (RSV) isolated from HIV-exposed-uninfected and unexposed infants in South Africa during 2015-2017. *Influenza Other Respir Viruses.* 2020;14(4):403-411.

73. Gieles NC, Mutsaerts E, Kwatra G, Bont L, Cutland CL, Jones S, Moultrie A, Madhi SA, **Nunes MC**. Measles seroprevalence in pregnant women in Soweto, South Africa: a nested cohort study. *Clin Microbiol Infect.* 2020;26(4):515 e511-515 e514.

74. Gieles NC, Mutsaerts E, Kwatra G, Bont L, Cutland CL, Jones S, Moultrie A, Madhi SA, **Nunes MC**. Rubella seroprevalence in pregnant women living with and without HIV in Soweto, South Africa. *Int J Infect Dis.* 2020;91:255-260.

75. Dhar N, Kwatra G, **Nunes MC**, Cutland C, Izu A, Nachbagauer R, Krammer F, Madhi SA. Hemagglutinin Stalk Antibody Responses Following Trivalent Inactivated Influenza Vaccine Immunization of Pregnant Women and Association With Protection From Influenza Virus Illness. *Clin Infect Dis.* 2020 Aug 14;71(4):1072-1079.

76. Clark DR, Omer SB, Tapia MD, **Nunes MC**, Cutland CL, Tielsch JM, Wairagkar N, Madhi SA; for BMGF Supported Maternal Influenza Immunization Trials Investigators Group. Influenza or Meningococcal Immunization During Pregnancy and Mortality in Women and Infants: A Pooled Analysis of Randomized Controlled Trials. *Pediatr Infect Dis J.* 2020 Jul;39(7):641-644.

77. Mutsaerts EAML, **Nunes MC**, Bhikha S, Ikulinda BT, Boyce W, Jose L, Koen A, Moultrie A, Cutland CL, Grobbee DE, Klipstein-Grobusch K, Madhi SA. Immunogenicity and Safety of an Early Measles Vaccination Schedule at 6 and 12 Months of Age in Human Immunodeficiency Virus (HIV)-Unexposed and HIV-Exposed, Uninfected South African Children. *J Infect Dis*. 2019 Sep 26;220(9):1529-1538.
78. Simões EAF, **Nunes MC**, Carosone-Link P, Madimabe R, Ortiz JR, Neuzil KM, Klugman KP, Cutland CL, Madhi SA; Matflu Team. Trivalent influenza vaccination randomized control trial of pregnant women and adverse fetal outcomes. *Vaccine*. 2019 Aug 23;37(36):5397-5403.
79. Li Y, Reeves RM, Wang X, Bassat Q, Brooks WA, Cohen C, Moore DP, **Nunes M**, Rath B, Campbell H, Nair H; RSV Global Epidemiology Network; RESCEU investigators. Global patterns in monthly activity of influenza virus, respiratory syncytial virus, parainfluenza virus, and metapneumovirus: a systematic analysis. *Lancet Glob Health*. 2019 Aug;7(8):e1031-e1045.
80. Jalbert E, Williamson KM, Kroehl ME, Johnson MJ, Cutland C, Madhi SA, **Nunes MC**, Weinberg A. HIV-Exposed Uninfected Infants Have Increased Regulatory T Cells That Correlate With Decreased T Cell Function. *Front Immunol*. 2019 Mar 26;10:595.
81. Cuningham W, Geard N, Fielding JE, Braat S, Madhi SA, **Nunes MC**, Christian LM, Lin SY, Lee CN, Yamaguchi K, Bisgaard H, Chawes B, Chao AS, Blanchard-Rohner G, Schlaudecker EP, Fisher BM, McVernon J, Moss R. Optimal timing of influenza vaccine during pregnancy: A systematic review and meta-analysis. *Influenza Other Respir Viruses*. 2019 Sep;13(5):438-452.
82. Baselga-Moreno V, Trushakova S, McNeil S, Sominina A, **Nunes MC**, Draganescu A, Unal S, Koul P, Kyncl J, Zhang T, Kvatbayeva A, Ben-Salah A, Burtseva E, Puig-Barberà J, Díez-Domingo J; Global Influenza Hospital Surveillance Network (GIHSN). Influenza epidemiology and influenza vaccine effectiveness during the 2016-2017 season in the Global Influenza Hospital Surveillance Network (GIHSN). *BMC Public Health*. 2019 May 2;19(1):487.
83. Simani OE, Izu A, **Nunes MC**, Violari A, Cotton MF, Van Niekerk N, Adrian PV, Madhi SA. Effect of HIV exposure and timing of antiretroviral therapy initiation on immune memory responses to diphtheria, tetanus, whole cell pertussis and hepatitis B vaccines. *Expert Rev Vaccines*. 2019 Jan;18(1):95-104.
84. Jallow S, Agosti Y, Kgagudi P, Vandecar M, Cutland CL, Simoes EAF, **Nunes MC**, Suchard MS, Madhi SA. Impaired Transplacental Transfer of Respiratory Syncytial Virus-neutralizing Antibodies in Human Immunodeficiency Virus-infected Versus -uninfected Pregnant Women. *Clin Infect Dis*. 2019 Jun 18;69(1):151-154.
85. Mutsaerts EAML, **Nunes MC**, van Rijswijk MN, Klipstein-Grobusch K, Otjombe K, Cotton MF, Violari A, Madhi SA. Measles Immunity at 4.5 Years of Age Following Vaccination at 9 and 15-18 Months of Age Among Human Immunodeficiency Virus (HIV)-infected, HIV-exposed-uninfected, and HIV-unexposed Children. *Clin Infect Dis*. 2019 Aug 1;69(4):687-696.
86. **Nunes MC**, Weinberg A, Cutland CL, Jones S, Wang D, Dighero-Kemp B, Levine MZ, Wairagkar N, Madhi SA. Neutralization and hemagglutination-inhibition antibodies following influenza vaccination of HIV-infected and HIV-uninfected pregnant women. *PLoS One*. 2018 Dec 31;13(12):e0210124.

87. **Nunes MC**, Cutland CL, Madhi SA. Influenza Vaccination during Pregnancy and Protection against Pertussis. *N Engl J Med*. 2018 Mar 29;378(13):1257-1258.
88. Mazur NI, Higgins D, **Nunes MC**, Melero JA, Langedijk AC, Horsley N, Buchholz UJ, Openshaw PJ, McLellan JS, Englund JA, Mejias A, Karron RA, Simões EA, Knezevic I, Ramilo O, Piedra PA, Chu HY, Falsey AR, Nair H, Kragten-Tabatabaie L, Greenough A, Baraldi E, Papadopoulos NG, Vekemans J, Polack FP, Powell M, Satav A, Walsh EE, Stein RT, Graham BS, Bont LJ; Respiratory Syncytial Virus Network (ReSVINET) Foundation. The respiratory syncytial virus vaccine landscape: lessons from the graveyard and promising candidates. *Lancet Infect Dis*. 2018 Oct;18(10):e295-e311.
89. Mutsaerts EAML, **Nunes MC**, van Rijswijk MN, Klipstein-Grobusch K, Grobbee DE, Madhi SA. Safety and Immunogenicity of Measles Vaccination in HIV-Infected and HIV-Exposed Uninfected Children: A Systematic Review and Meta-Analysis. *EClinicalMedicine*. 2018;1:28-42.
90. Madhi SA, **Nunes MC**. Experience and challenges on influenza and pertussis vaccination in pregnant women. *Hum Vaccin Immunother*. 2018. 14(9): 2183-2188.
91. Olwagen CP, Adrian PV, **Nunes MC**, Madhi SA. Evaluation of the association of pneumococcal conjugate vaccine immunization and density of nasopharyngeal bacterial colonization using a multiplex quantitative polymerase chain reaction assay. *Vaccine*. 2018;36(23):3278-85.
92. Omer SB, Clark DR, Aqil AR, Tapia MD, **Nunes MC**, Kozuki N, Steinhoff MC, Madhi SA, Wairagkar N and for BSMITIG. Maternal Influenza Immunization and Prevention of Severe Clinical Pneumonia in Young Infants: Analysis of Randomized Controlled Trials Conducted in Nepal, Mali, and South Africa. *Pediatr Infect Dis J*. 2018 May;37(5):436-440.
93. **Nunes MC**, Madhi SA. Prevention of influenza-related illness in young infants by maternal vaccination during pregnancy. *F1000Res*. 2018;7:122.
94. **Nunes MC**, Madhi SA. Influenza vaccination during pregnancy for prevention of influenza confirmed illness in the infants: A systematic review and meta-analysis. *Hum Vaccin Immunother*. 2018;14(3):758-66.
95. Obando-Pacheco P, Justicia-Grande AJ, Rivero-Calle I, Rodríguez-Tenreiro C, Sly P, Ramilo O, Mejias A, Baraldi E, Papadopoulos NG, Nair H, **Nunes MC**, Kragten-Tabatabaie L, Heikkinen T, Greenough A, Stein RT, Manzoni P, Bont L, Martínón-Torres F. Respiratory Syncytial Virus Seasonality: A Global Overview. *J Infect Dis*. 2018 Apr 11;217(9):1356-1364.
96. Madhi SA, Cutland CL, Downs S, Jones S, van Niekerk N, Simoes EAF and **Nunes MC**. Burden of Respiratory Syncytial Virus Infection in South African Human Immunodeficiency Virus (HIV)-Infected and HIV-Uninfected Pregnant and Postpartum Women: A Longitudinal Cohort Study. *Clin Infect Dis*. 2018;66(11):1658-65.
97. Olwagen CP, Adrian PV, **Nunes MC**, Groome MJ, Cotton MF, Violari A and Madhi SA. Use of Multiplex Quantitative PCR To Evaluate the Impact of Pneumococcal Conjugate Vaccine on Nasopharyngeal Pneumococcal Colonization in African Children. *mSphere*. 2017;2(6).
98. Nzenze SA, Madhi SA, Shiri T, Klugman KP, de Gouveia L, Moore DP, Karstaedt AS, Tempia S, **Nunes MC** and von Gottberg A. Imputing the Direct and Indirect Effectiveness of Childhood Pneumococcal Conjugate Vaccine Against Invasive Pneumococcal Disease by

Surveying Temporal Changes in Nasopharyngeal Pneumococcal Colonization. *Am J Epidemiol.* 2017 Aug 15;186(4):435-444.

99. **Nunes MC**, Cutland CL, Jones S, Downs S, Weinberg A, Ortiz JR, Neuzil KM, Simoes EAF, Klugman KP and Madhi SA. Efficacy of maternal influenza vaccination against all-cause lower respiratory tract infection hospitalizations in young infants: Results from a randomized controlled trial. *Clin Infect Dis.* 2017;65(7):1066-71.
100. Madhi SA, **Nunes MC**, Weinberg A, Kuwanda L, Hugo A, Jones S, van Niekerk N, Ortiz JR, Neuzil KM, Klugman KP, Simoes EAF, Cutland CL and Maternal Flu Trial T. Contribution of Serologic Assays in the Evaluation of Influenza Virus Infection Rates and Vaccine Efficacy in Pregnant Women: Report From Randomized Controlled Trials. *Clin Infect Dis.* 2017;64(12):1773-9.
101. Jallow S, Madhi SA, Madimabe R, Sipambo N, Violari A, Kala U, Petersen K, Naidoo S, Verwey C, Moore DP and **Nunes MC**. Immunogenicity of 13-valent pneumococcal conjugate vaccine among children with underlying medical conditions. *Vaccine.* 2017;35(34):4321-9.
102. Heath PT, Culley FJ, Jones CE, Kampmann B, Le Doare K, **Nunes MC**, Sadarangani M, Chaudhry Z, Baker CJ and Openshaw PJM. Group B streptococcus and respiratory syncytial virus immunisation during pregnancy: a landscape analysis. *Lancet Infect Dis.* 2017;17(7):e223-e34.
103. Dangor Z, **Nunes MC**, Kwatra G, Lala SG and Madhi SA. Vaccination of HIV-infected pregnant women: implications for protection of their young infants. *Trop Dis Travel Med Vaccines.* 2017;3:1.
104. Soofie N, **Nunes MC**, Kgagudi P, van Niekerk N, Makgobo T, Agosti Y, Hwinya C, Pathirana J and Madhi SA. The Burden of Pertussis Hospitalization in HIV-Exposed and HIV-Unexposed South African Infants. *Clin Infect Dis.* 2016;63(suppl 4):S165-S73.
105. **Nunes MC**, Soofie N, Downs S, Tebeila N, Mudau A, de Gouveia L and Madhi SA. Comparing the Yield of Nasopharyngeal Swabs, Nasal Aspirates, and Induced Sputum for Detection of *Bordetella pertussis* in Hospitalized Infants. *Clin Infect Dis.* 2016;63(suppl 4):S181-S6.
106. **Nunes MC**, Downs S, Jones S, van Niekerk N, Cutland CL and Madhi SA. *Bordetella pertussis* Infection in South African HIV-Infected and HIV-Uninfected Mother-Infant Dyads: A Longitudinal Cohort Study. *Clin Infect Dis.* 2016;63(suppl 4):S174-S80.
107. **Nunes MC**, Cutland CL, Jones S, Hugo A, Madimabe R, Simoes EA, Weinberg A, Madhi SA and Maternal Flu Trial T. Duration of Infant Protection Against Influenza Illness Conferred by Maternal Immunization: Secondary Analysis of a Randomized Clinical Trial. *JAMA Pediatr.* 2016;170(9):840-7.
108. **Nunes MC**, Aqil AR, Omer SB and Madhi SA. The Effects of Influenza Vaccination during Pregnancy on Birth Outcomes: A Systematic Review and Meta-Analysis. *Am J Perinatol.* 2016;33(11):1104-14.
109. Mutsaerts E, Madhi SA, Cutland CL, Jones S, Hugo A, Trenor S, Treurnicht FK, Klipstein-Grobusch K, Weinberg A and **Nunes MC**. Influenza vaccination of pregnant women protects them over two consecutive influenza seasons in a randomized controlled trial. *Expert Rev Vaccines.* 2016;15(8):1055-62.

110. Madhi SA and **Nunes MC**. The potential impact of pneumococcal conjugate vaccine in Africa: Considerations and early lessons learned from the South African experience. *Hum Vaccin Immunother.* 2016;12(2):314-25.
111. Nzenze SA, von Gottberg A, Shiri T, van Niekerk N, de Gouveia L, Violari A, **Nunes MC** and Madhi SA. Temporal Changes in Pneumococcal Colonization in HIV-infected and HIV-uninfected Mother-Child Pairs Following Transitioning From 7-valent to 13-valent Pneumococcal Conjugate Vaccine, Soweto, South Africa. *J Infect Dis.* 2015 Oct 1;212(7):1082-92.
112. **Nunes MC** and Madhi SA. Review on the effects of influenza vaccination during pregnancy on preterm births. *Hum Vaccin Immunother.* 2015;11(11):2538-48.
113. **Nunes MC**, Jones SA, Groome MJ, Kuwanda L, Van Niekerk N, von Gottberg A, de Gouveia L, Adrian PV and Madhi SA. Acquisition of *Streptococcus pneumoniae* in South African children vaccinated with 7-valent pneumococcal conjugate vaccine at 6, 14 and 40 weeks of age. *Vaccine.* 2015;33(5):628-34.
114. **Nunes MC**, Cutland CL, Dighero B, Bate J, Jones S, Hugo A, van Niekerk N, Kuwanda L, Izu A, Weinberg A, Madhi SA and Matflu T. Kinetics of Hemagglutination-Inhibiting Antibodies Following Maternal Influenza Vaccination Among Mothers With and Those Without HIV Infection and Their Infants. *J Infect Dis.* 2015 Dec 15;212(12):1976-87.
115. Madhi SA, Izu A, **Nunes MC**, Violari A, Cotton MF, Jean-Philippe P, Klugman KP, von Gottberg A, van Niekerk N, Adrian PV; CIPRA 4 team. Longitudinal study on *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* nasopharyngeal colonization in HIV-infected and -uninfected infants vaccinated with pneumococcal conjugate vaccine. *Vaccine.* 2015 May 28;33(23):2662-9.
116. Madhi SA, Govender N, Dayal K, Devadiga R, Van Dyke MK, van Niekerk N, Cutland CL, Adrian PV, **Nunes MC**. Bacterial and Respiratory Viral Interactions in the Etiology of Acute Otitis Media in HIV-infected and HIV-uninfected South African Children. *Pediatr Infect Dis J.* 2015 Jul;34(7):753-60.
117. Nzenze SA, Shiri T, **Nunes MC**, Klugman KP, Kahn K, Twine R, de Gouveia L, von Gottberg A and Madhi SA. Temporal association of infant immunisation with pneumococcal conjugate vaccine on the ecology of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* nasopharyngeal colonisation in a rural South African community. *Vaccine.* 2014;32(42):5520-30.
118. **Nunes MC**, Kuschner Z, Rabede Z, Madimabe R, Van Niekerk N, Moloi J, Kuwanda L, Rossen JW, Klugman KP, Adrian PV and Madhi SA. Clinical epidemiology of bocavirus, rhinovirus, two polyomaviruses and four coronaviruses in HIV-infected and HIV-uninfected South African children. *PLoS One.* 2014;9(2):e86448.
119. **Nunes MC**, Kuschner Z, Rabede Z, Cutland CL, Madimabe R, Kuwanda L, Klugman KP, Adrian PV and Madhi SA. Polyomaviruses-associated respiratory infections in HIV-infected and HIV-uninfected children. *J Clin Virol.* 2014 Dec;61(4):571-8.
120. Madhi SA, Cutland CL, Kuwanda L, Weinberg A, Hugo A, Jones S, Adrian PV, van Niekerk N, Treurnicht F, Ortiz JR, Venter M, Violari A, Neuzil KM, Simoes EA, Klugman KP, **Nunes MC** and Maternal Flu Trial T. Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med.* 2014;371(10):918-31.

121. Dangor Z, Izu A, Moore DP, **Nunes MC**, Solomon F, Beylis N, von Gottberg A, McAnerney JM and Madhi SA. Temporal association in hospitalizations for tuberculosis, invasive pneumococcal disease and influenza virus illness in South African children. *PLoS One*. 2014;9(3):e91464.
122. Simani OE, Adrian PV, Violari A, Kuwanda L, Otwombe K, **Nunes MC**, Cotton MF, Madhi SA. Effect of in-utero HIV exposure and antiretroviral treatment strategies on measles susceptibility and immunogenicity of measles vaccine. *AIDS*. 2013;27(10):1583-91.
123. Shiri T, **Nunes MC**, Adrian PV, Van Niekerk N, Klugman KP, Madhi SA. Interrelationship of *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* colonization within and between pneumococcal-vaccine naïve mother-child dyads. *BMC Infect Dis*. 2013 Oct 17;13:483.
124. Shiri T, Auranen K, **Nunes MC**, Adrian PV, van Niekerk N, de Gouveia L, von Gottberg A, Klugman KP, Madhi SA. Dynamics of pneumococcal transmission in vaccine-naive children and their HIV-infected or HIV-uninfected mothers during the first 2 years of life. *Am J Epidemiol*. 2013 Dec 1;178(11):1629-37.
125. Nzenze SA, Shiri T, **Nunes MC**, Klugman KP, Kahn K, Twine R, de Gouveia L, von Gottberg A, Madhi SA. Temporal changes in pneumococcal colonization in a rural African community with high HIV prevalence following routine infant pneumococcal immunization. *Pediatr Infect Dis J*. 2013 Nov;32(11):1270-8.
126. **Nunes MC**, Shiri T, van Niekerk N, Cutland CL, Groome MJ, Koen A, von Gottberg A, de Gouveia L, Klugman KP, Adrian PV, Madhi SA. Acquisition of *Streptococcus pneumoniae* in pneumococcal conjugate vaccine-naïve South African children and their mothers. *Pediatr Infect Dis J*. 2013 May;32(5):e192-205.
127. **Nunes MC**, Madhi SA. Review of a new fully liquid, hexavalent vaccine: Hexaxim. *Expert Opin Biol Ther*. 2013 Apr;13(4):575-93.
128. Dangor Z, Izu A, Hillier K, Solomon F, Beylis N, Moore DP, **Nunes MC**, Madhi SA. Impact of the antiretroviral treatment program on the burden of hospitalization for culture-confirmed tuberculosis in South African children: a time-series analysis. *Pediatr Infect Dis J*. 2013 Sep;32(9):972-7.
129. **Nunes MC** and Madhi SA. Safety, immunogenicity and efficacy of pneumococcal conjugate vaccine in HIV-infected individuals. *Hum Vaccin Immunother*. 2012;8(2):161-73.
130. Gomes MG, Aguas R, Lopes JS, **Nunes MC**, Rebelo C, Rodrigues P, Struchiner CJ. How host heterogeneity governs tuberculosis reinfection? *Proc Biol Sci*. 2012.
131. **Nunes MC**, von Gottberg A, de Gouveia L, Cohen C, Moore DP, Klugman KP, Madhi SA. The impact of antiretroviral treatment on the burden of invasive pneumococcal disease in South African children: a time series analysis. *AIDS*. 2011;25(4):453-62.
132. **Nunes MC**, von Gottberg A, de Gouveia L, Cohen C, Kuwanda L, Karstaedt AS, Klugman KP, Madhi SA. Persistent high burden of invasive pneumococcal disease in South African HIV-infected adults in the era of an antiretroviral treatment program. *PLoS One*. 2011;6(11):e27929.
133. **Nunes MC** and Madhi SA. Review on the immunogenicity and safety of PCV-13 in infants and toddlers. *Expert Rev Vaccines*. 2011;10(7):951-80.

134. van Noort SP, **Nunes MC**, Weedall GD, Hviid L, Gomes MG. Immune selection and within-host competition can structure the repertoire of variant surface antigens in *Plasmodium falciparum*--a mathematical model. *PLoS One*. 2010;5(3):e9778.
135. **Nunes MC**, Okada M, Scheidig-Benatar C, Cooke BM, Scherf A. *Plasmodium falciparum* FIKK kinase members target distinct components of the erythrocyte membrane. *PLoS One*. 2010;5(7):e11747.
136. **Nunes MC**, Sterkers Y, Gamain B, Scherf A. Investigation of host factors possibly enhancing the emergence of the chondroitin sulfate A-binding phenotype in *Plasmodium falciparum*. *Microbes Infect*. 2008;10(8):928-32.
137. **Nunes MC** and Scherf A. *Plasmodium falciparum* during pregnancy: a puzzling parasite tissue adhesion tropism. *Parasitology*. 2007;134(Pt 13):1863-9.
138. **Nunes MC**, Goldring JP, Doerig C, Scherf A. A novel protein kinase family in *Plasmodium falciparum* is differentially transcribed and secreted to various cellular compartments of the host cell. *Mol Microbiol*. 2007;63(2):391-403.
139. Lopez-Rubio JJ, Gontijo AM, **Nunes MC**, Issar N, Hernandez Rivas R, Scherf A. 5' flanking region of var genes nucleate histone modification patterns linked to phenotypic inheritance of virulence traits in malaria parasites. *Mol Microbiol*. 2007;66(6):1296-305.
140. Lin JH, Takano T, Arcuino G, Wang X, Hu F, Darzynkiewicz Z, **Nunes M**, Goldman SA, Nedergaard M. Purinergic signaling regulates neural progenitor cell expansion and neurogenesis. *Dev Biol*. 2007 Feb 1;302(1):356-66.
141. Viebig NK, **Nunes MC**, Scherf A, Gamain B. The human placental derived BeWo cell line: a useful model for selecting *Plasmodium falciparum* CSA-binding parasites. *Exp Parasitol*. 2006;112(2):121-5.
142. Windrem MS, **Nunes MC**, Rashbaum WK, Schwartz TH, Goodman RA, McKhann G 2nd, Roy NS, Goldman SA. Fetal and adult human oligodendrocyte progenitor cell isolates myelinate the congenitally dysmyelinated brain. *Nat Med*. 2004 Jan;10(1):93-7.
143. **Nunes MC**, Roy NS, Keyoung HM, Goodman RR, McKhann G 2nd, Jiang L, Kang J, Nedergaard M, Goldman SA. Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain. *Nat Med*. 2003 Apr;9(4):439-47.
144. Windrem MS, Roy NS, Wang J, **Nunes M**, Benraiss A, Goodman R, McKhann GM 2nd, Goldman SA. Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated lesions of the rat brain. *J Neurosci Res*. 2002 Sep 15;69(6):966-75.
146. Neves AR, Ramos A, **Nunes MC**, Kleerebezem M, Hugenholtz J, de Vos WM, Almeida J, Santos H. In vivo nuclear magnetic resonance studies of glycolytic kinetics in *Lactococcus lactis*. *Biotechnol Bioeng*. 1999 Jul 20;64(2):200-12.
147. Antonio CM, **Nunes MC**, Refsum H, Abraham AK. A novel pathway for the conversion of homocysteine to methionine in eukaryotes. *Biochem J*. 1997 Nov 15;328 ( Pt 1)(Pt 1):165-70.



## Meeting reports

1. Abusrewil S, Algeer A, Aljifri A, Al Slail F, Andrew MK, Awad Tag Eldin M, Al Awaidy S, Ben Alaya N, Ben Khelil J, Dbaibo G, Derrar F, Elahmer O, Ghosn N, Gabriel G, Grasso C, Hassan M, Hirve S, Mirza YK, Rateb YM, Nourlil J, **Nunes MC**, Omaima I, Malande OO, Saadatian-Elahi M, Sanchez-Picot V, Sk Mamunur Rahman M, Tarraf H, Walaza S. Influenza surveillance in Middle East, North, East and South Africa: Report of the 8th MENA Influenza Stakeholders Network. *Influenza Other Respir Viruses*. 2019 May;13(3):298-304.
2. Nair H, Ramilo O, Eichler I, Pelfrene E, Mejias A, Polack FP, Pouwels KB, Langley JM, **Nunes M**, van der Maas N, Kragten–Tabatabaie L, Baraldi E, Heikkinen T, Fauroux B, Sharland M, Park C, Manzoni P, Papadopoulos NG, Martínón–Torres F, Stein R, Bont L; in collaboration with Respiratory Syncytial Virus Network (ReSViNET). Meeting Report: Harmonization of RSV therapeutics – from design to performance. *J Glob Health*. 2016 Jun;6(1):010205.

## Invited commentaries and letters

1. Vanhems P, **Nunes MC**. Further Cautions When Modeling the Public Health Impact of Infectious Diseases. *Am J Public Health*. 2022;112(11):e1-e2.
2. Jones S, **Nunes MC**. Beyond COVID-19: Equitable epidemiology for studying the impact of maternal infections on neonatal mortality and morbidity. *Paediatr Perinat Epidemiol*. 2022 Jul;36(4):553-5.
3. Verwey C, **Nunes MC**. RSV lower respiratory tract infection and lung health in the first 2 years of life. *Lancet Glob Health*. 2020;8(10):e1247-e8.
4. Cohen C, **Nunes MC**. Vaccinating Mothers to Protect Their Babies Against Influenza. *J Infect Dis*. 2020;221(1):5-7.
5. Madhi SA, **Nunes MC**, Cutland CL: Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med*. 2014, 371(24):2340.

## Book chapters

1. **Nunes MC**, Lemos SR. Influenza. In: *Maternal Immunisation, The Continuous Textbook of Women's Medicine Series, Obstetric Module, Volume 17*. Khalil A, Munoz F (Eds.). Global Library of Women's Medicine (2023).
2. Fell D, Tapia M, **Nunes MC**. Influenza. In: *Maternal Immunization*. Leuridan E, Nunes MC, Jones C (Eds.). Elsevier, Academic Press (2019).
3. Leuridan E, **Nunes MC**, Jones C. Conclusion. In: *Maternal Immunization*. Leuridan E, Nunes MC, Jones C (Eds.). Elsevier, Academic Press (2019).

4. **Nunes MC**, Madhi SA. Pneumococcal conjugate vaccine in immunocompromised individuals. In: Pneumococcal Conjugate Vaccines. Rijkers GT (Ed.). Future Medicine Ltd, London, UK (2012).
5. Windrem M, Roy N, **Nunes M**, Goldman, SA. Isolation, selection, and use of adult human oligodendrocyte progenitor cells. In: Neural Stem Cells from Brain and Spinal Cord Repair. Zigova T, Snyder E, Sanberg P (Eds.). Humana Press Inc, Totowa, NJ (2003).

### Edited books

Leuridan E, **Nunes MC**, Jones C. Maternal Immunization. Elsevier, Academic Press (2019).

### Invited presentations

42nd Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 20-24 May 2024, Copenhagen, Denmark. “What do we know about COVID-19 vaccination in pregnancy?”.

13th Meeting of the International Society of Pneumonia & Pneumococcal Diseases (ISPPD-13), 17-20 March 2024, Cape Town, South Africa. “Immunization of Pregnant Individuals to Reduce Infectious Disease Early in Life”.

Vaccinology Symposium, 6-8 October 2023, Johannesburg, South Africa. “HIV and vaccination in pregnancy (Tdap & IIV) and Clinical trial update” (virtual presentation).

11th ICCN – International Conference on Clinical Neonatology, 7-9 July 2023, Turin, Italy. “Overview of future vaccines for respiratory viruses in infancy”.

40th National Congress of the SASOG – South African Society of Obstetricians and Gynaecologists, 28-30 November 2022, Cape Town, South Africa. “Vaccination among HIV-infected and HIV-uninfected pregnant women”.

10th ICCN – International Conference on Clinical Neonatology, 21-23 May 2022, Turin (hybrid), Italy. “An overview and update about maternal vaccines and maternal vaccination strategies”.

International Society for Influenza and other Respiratory Virus Diseases (ISIRV)-WHO Virtual Conference, 19-21 October 2021, virtual. “Preventing RVIs in Pregnant Women and Infants”.

Light on Vax 4, 28 June-1 July 2021, virtual. “Estrategias de inmunización frente al VRS”.

39th Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 24-29 May 2021, virtual. RSV symposium: “RSV – an annual epidemic”.

39th Annual Meeting of the European Society for Paediatric Infectious Diseases (ESPID), 24-29 May 2021, virtual. Pediatric Pneumococcal Disease Symposium: “COVID-19 and Pneumococcal Disease in Children”.

Health in a Global context: “Advocating for Global Health and SDGs: creating synergies for Global Health and SDG Research and Innovation” Meeting, 6 October 2020, virtual. “Global Health as a multisectorial research effort: the COVID-19 response”.

Global Experts’ Meeting (GEM) 2020, 3-4 September 2020, virtual. “Maternal RSV vaccination”.

13th Congress of the European Society of Gynecology, 13-19 October 2019, Vienna, Austria. “Why vaccinate pregnant women?”.

Options X for the Control of Influenza, 28 August-1 September 2019, Singapore. “Impact of Influenza Vaccination during Pregnancy on the Mother, Fetus, and Infant”.

4th Southern African HIV Clinicians Society Conference, 24-27 October 2018, Midrand, South Africa. “Influenza vaccines”.

10th World Congress of the World Society for Pediatric Infectious Diseases (WSPID), 2-5 December 2017, Shenzhen, China. “Impact of maternal vaccination on mother, fetus and infant”.

4th ReSViNET Conference on RSV therapeutics, 29 November-1 December 2017, Malaga, Spain. “Clinical endpoints in trials of RSV vaccines in pregnant women: study design, assessment of safety and effectiveness”.

18th World Vaccine Congress, 10-12 October 2017, Barcelona, Spain. “Landscape analysis of RSV vaccine development and future implications”.

6th European Scientific Working group on Influenza (ESWI) Conference, 10-13 September 2017, Riga, Latvia. “Maternal immunization against influenza: benefits to mother and child”.

3rd ReSViNET High-Level Expert Meeting, 8-10 March 2017, Amsterdam, Netherlands. “Respiratory tract infections in HIV-infected and HIV-exposed children”.

6th International Congress of Union of European Neonatal and Perinatal Societies (UENPS), 23-25 November 2016, Valencia, Spain. “The current state of the maternal immunization during pregnancy”.

2nd ReSViNET High-Level Expert Meeting, 2-3 March 2016, Zeist, Netherlands. “Clinical endpoints in trials of RSV vaccines in pregnant women: study design issues, assessment of safety and effectiveness”.

5th South African Immunology Society Conference, 6-9 March 2016, Muldersdrift, South Africa. “Influenza vaccination during pregnancy and transplacental antibody transfer”.

9th World Congress of the World Society for Pediatric Infectious Diseases (WSPID), 18-21 November 2015, Rio de Janeiro, Brazil. “Influenza immunisation of pregnant women”.

Pertussis: biology, epidemiology and prevention, 11-13 November 2015, Les Pensières conference center, Annecy, France. “Epidemiology of Pertussis in Africa. Maternal immunization as a possible strategy for prevention”.

13th Global Experts’ Meeting (GEM), 6-8 October 2015, Rome, Italy. “Maternal Immunisation – Impact on Childhood Respiratory Disease”.

2nd International Conference of the African Society for Laboratory Medicine (ASLM), 30 November–4 December 2014, Cape Town, South Africa. “Maternal Influenza Vaccination an Update from Africa”.

2014 Grand Challenges Meeting, 5-8 October 2014, Seattle, USA. “Influenza vaccination of HIV-uninfected and HIV-infected pregnant women and protection of their infants”.

5th Federation of Infectious Diseases Societies of Southern Africa (FIDSSA) Conference. 10-12 October 2013, Drakensberg, South Africa. “Maternal Immunization”.

African Influenza and Emerging Respiratory Virus Preparedness Meeting. 4 September 2013, Cape Town, South Africa. “Influenza in pregnancy”.

## Organizer, Faculty

CERP modelling workshop in infectious diseases, 12-13 July 2023, Lyon, France. Organizer.

7th European Scientific Working group on Influenza (ESWI) Conference, ESWI2020 6-9 December 2020, virtual. Scientific Programme Committee.

5th ReSViNET Global Conference on Novel RSV Preventive and Therapeutic Interventions, RSVVW'19, 12-14 November 2019, Accra, Ghana. Organizer.

Course on viral respiratory infections in neonates and infants, 21-22 September 2016, Turin, Italy. Organizer and Faculty. Presentation on: "Safety and efficacy of maternal vaccines against influenza and pertussis".

Workshop on Evolution in Health and Disease, September 2008, Gulbenkian Institute of Science, Oeiras, Portugal. Organizer.

## Courses attended

'Leadership through Trust in Disruptive Times' programme, March - July 2021, Wits Business School, University of the Witwatersrand, Johannesburg, South Africa.

Supervisors course, 17 - 20 July 2017, Health Sciences Research Office, University of the Witwatersrand, Johannesburg, South Africa.

13th Advance Vaccinology Course, 14 – 25 May, 2012, Les Pensières, Annecy, France. Full scholarship awarded.

Good Clinical Practice, 27 – 28 January 2011, Wits Health Consortium, University of the Witwatersrand, Johannesburg, South Africa.

European Course on Tropical Epidemiology, 25 August – 12 September 2008, Department of Tropical Hygiene and Public Health, University of Heidelberg, Germany.

## Teaching experience

Master LIVE in international vaccinology (Erasmus Mundus Joint Master "Leading International Vaccinology Education"). October 2023, Université Claude Bernard Lyon 1, Lyon, France. Lecturer: Epidemiology.

Masters in Vaccinology. June - September 2022, University of the Witwatersrand, Johannesburg, South Africa. Lecturer and coordinator of Module 6: Advanced Vaccinology.

3rd African Advanced Vaccinology Course (Afro-Advac). 13-22 September 2021, Muldersdrift, South Africa. Lecturer.

Princípios da Escrita Científica. July 2021, Calouste Gulbenkian Foundation, International Cooperation, Portugal. Virtual workshop. Lecturer and coordinator.

Masters in Vaccinology. July - October 2021, University of the Witwatersrand, Johannesburg, South Africa. Lecturer and coordinator of Module 6: Advanced Vaccinology.

Princípios da Escrita Científica. December 2020, Calouste Gulbenkian Foundation, International Cooperation, Portugal. Virtual workshop. Lecturer and coordinator.

Masters in Vaccinology. July - November 2019, University of the Witwatersrand, Johannesburg, South Africa. Lecturer and coordinator of Module 6: Advanced Vaccinology.

Summer School on 'Child Health: A Global Perspective'. 20-24 August 2018, Utrecht, the Netherlands. Guest lecturer.

2nd Afro-Advac. 3-12 September 2018, Muldersdrift, South Africa. Organizer and lecturer.

1st Afro-Advac. 19-28 September 2016, Muldersdrift, South Africa. Organizer and lecturer.

Biochemistry. January – May 1997, Pre-clinical Institute, Medical College of Bergen University, Bergen, Norway. Teaching assistant in biochemistry.

## Fellowships

2018-2019 Wits Health Consortium, Johannesburg, South Africa, Activating Academic Entrepreneurship: Great Leap Forward Fellowship.

2012-2013 University of the Witwatersrand, Johannesburg, South Africa, SPARC Prestigious Post-doctoral Fellowship.

2010-2012 University of the Witwatersrand, Johannesburg, South Africa, Post-doctoral Fellowship.

2009-2010 Fundação para a Ciência e a Tecnologia, Portugal, Post-doctoral Fellowship.

2005-2007 Human Frontiers Science Program, Post-doctoral Fellowship.

- 2004 European Molecular Biology Organization (EMBO), Post-doctoral Long-term Fellowship.
- 1998-2003 Fundação Calouste Gulbenkian and Fundação para a Ciência e a Tecnologia, Gulbenkian PhD program in Biology and Medicine PhD fellowship.
- 1997-1998 Fundação para a Ciência e a Tecnologia, Portugal, Young Researcher Fellowship.
- 1996-1997 European student exchanging program, Erasmus Fellowship.

## Awards

- 2019 Travel Bursary Award, 5th International Neonatal & Maternal Immunization Symposium, Vancouver, Canada.
- 2013 Meeting Travel Award, World Congress of the World Society for Pediatric Infectious Diseases, Cape Town, South Africa.
- 2006 Postdoctoral Presentation Prize, International Congress of Parasitology, Glasgow, Scotland, UK.
- 2005 Nomination to participate in the Meeting of Nobel Laureates, Lindau, Germany.
- 1998 Accepted to the Gulbenkian PhD program in Biology and Medicine, Portugal.
- 1998 Gulbenkian Science Award (for project developed at IQTB), Portugal.

## Board and societies memberships

- 2023-present Chair of the scientific advisory committee. Global Influenza Hospital Surveillance Network.
- 2021-present Vice-president. Associação Viver a Ciência.
- 2020-present Editorial board member of the journal Pneumonia.
- 2019-present Board member, Treasurer. Middle East, Eurasia and Africa Influenza Stakeholders Network.

- 2019-2022 Committee member. Gavi Evaluation Advisory Committee.
- 2018-2022 Board member. Gavi, The Vaccine Alliance.
- 2018-2023 Scientific advisory committee member. Global Influenza Hospital Surveillance Network.
- 2017-2022 Member. European Society of Paediatric Infectious Diseases.
- 2016-present Board member. ReSViNET Foundation, Respiratory Syncytial Virus Network.
- 2016-present Member. The Global Pertussis Initiative.

### Technical consultations

- May 2024 RSV Vaccine (Maternal Indication) Medical Advisory Board. Pfizer Gulf.
- December 2023- Global RSV KOL Engagement Program. Steering Committee Member. Pfizer.
- April 2023 COVID-19 Expert Consultation. Sanofi.
- Aug 2021- Determining whether mass campaigns with fractional dose PCV10 would accelerate herd protection against pneumococcal transmission in Sub-Saharan Africa. Epicentre. Trial Steering Committee Member.
- April 2021- PROUD – Preventing Respiratory syncytial viruses in underdeveloped countries. Project Steering Committee Member.
- April 2021 RSV Passive Immunization: mAbs and maternal immunization. Sanofi.
- October 2020 RSV prevention for all infants. Sanofi Pasteur.
- 2019-2020 Maternal immunization uptake panel. Pfizer.
- January 2020 Pediatric Flu advisory board. Sanofi Pasteur.
- November 2019 Influenza advisory board (South Africa). Sanofi Pasteur.
- October 2019 Respiratory Syncytial Virus virtual advisory board (South Africa). Sanofi Pasteur.



April 2019 Stakeholder led research prioritization event on preterm birth. Preterm Birth Prevention and Management (PRIME), multi-country project funded by the National Institute for Health Research, UK.

June 2018 Roundtable meeting, Current global perspectives and remaining challenges of Influenza and Respiratory Syncytial Virus. Global Influenza Initiative.

September 2018 Guidelines on the quality, safety and efficacy of Respiratory Syncytial Virus. World Health Organization.

June 2017 Respiratory Syncytial Virus early phase advisory board. Pfizer.

December 2016 PCV13 Paediatric age expansion advisory board (South Africa). Pfizer.

November 2016 Roundtable meeting, Pertussis: The African perspective and remaining global challenges. Global Pertussis Initiative.

February 2016 Pertussis surveillance and mortality mini symposium. Bill & Melinda Gates Foundation.

August 2015 Pertussis interest group meeting (South Africa). Sanofi Pasteur.

July 2015 Roundtable meeting, GII. Global Influenza Initiative.

May 2015 Immunology research gaps related to maternal immunization. Bill & Melinda Gates Foundation.

March 2015 Maternal influenza immunization evidence and implementation. World Health Organization.

February 2013 International Pneumococcal Expert Panel. Pfizer.

January 2013 First WHO integrated meeting on development and clinical trials of Influenza vaccines that induce broadly protective and long-lasting immune responses. World Health Organization.

## Research funding (last 10 years)

Bill & Melinda Gates Foundation 2014-2016 \$779,384

Title: Burden of pertussis hospitalization in HIV-exposed and HIV-unexposed South African infants.

Bill & Melinda Gates Foundation 2015-2020 \$3,060,440

Title: Effectiveness of influenza vaccination of pregnant women in protecting against influenza associated hospitalization in young HIV-exposed and HIV-unexposed infants: a case control study.

Foundation for Influenza Epidemiology 2017-2022 €560,000

Title: Global Influenza Hospital Surveillance Network.

National Institute of Allergy and Infectious Diseases, National Institutes of Health, US (Grant awarded to our collaborator at the University of Colorado) 2017-2021 \$313,000

Title: Tregs, Gut Microbiome and Immune Responses in HIV-Exposed and Unexposed Infants.

MedImmune 2018 \$25,000

Title: RSV next-generation sequencing.

MedImmune 2018-2022 €101,500

Title: International Network for Optimal Resistance Monitoring of RSV (INFORM RSV).

National Institute of Health Research (NIHR), Global Health Research Unit on Mucosal Pathogens, UK 2020-2021 £92,113

Title: Molecular evaluation of the association of the vaginal microbiome and adverse pregnancy outcomes in South Africa.

Bill & Melinda Gates Foundation 2020-2021 \$630,490

Title: Investigation of hospitalized adults for SARS-Coronavirus–2 (COVID-19) associated pneumonia in a setting with high prevalence of HIV and Tuberculosis.

Bill & Melinda Gates Foundation 2020-2021 \$1,140,129

Title: SARS-CoV–2 infection among healthcare workers.

European & Developing Countries Clinical Trials Partnership 2020-2022 €499,938

Title: Surveillance among healthcare workers for SARS-Coronavirus–2 infection.

Pfizer 2020-2021 \$100,000

Title: Temporal changes of drug-resistant Streptococcus pneumoniae in South African children in the era of childhood pneumococcal immunization.

Bill & Melinda Gates Foundation 2021-2022 \$277,672

Title: RSV dynamics in the context of COVID - South Africa.

AstraZeneca 2021-2022 \$2,543,916

Title: Effectiveness of Covid-19 vaccination in Eswatini against SARS-CoV-2 associated hospitalization and death.

Sanofi 2022-2025 ZAR26,062,883

Title: Pertussis vaccination among HIV-infected and HIV-uninfected pregnant women.

Shape-Med Lyon 2023-2025 €113,000

Title: Extraction of symptoms from electronic health records to create automatic cohorts.

Agence Nationale de la Recherche/Sanofi Programme Chaire Industrielles  
2024-2027 €1,000,000

Title: Global burden of respiratory viruses with interest for vaccine development.

## Post-graduate Supervision

### PhD students

Alexander Malherbe, University of the Witwatersrand, 2013-2017.

Thesis: Cell-mediated and humoral immune responses to trivalent inactivated influenza vaccine in high-risk groups.

Eleonora Mutsaerts, University of the Witwatersrand, 2016-2020.

Thesis: Safety and immunogenicity of measles vaccine, varicella vaccine and hepatitis-A vaccine in HIV-exposed and HIV-unexposed South African children.

*Publications from the PhD work:*

Mutsaerts EAML, Nunes MC, Bhikha S, Ikulinda BT, Jose L, Koen A, Moultrie A, Grobbee DE, Klipstein-Grobusch K, Weinberg A, Madhi SA. Short-term immunogenicity and safety of hepatitis-A and varicella vaccines in HIV-exposed uninfected and HIV-unexposed South African children. *Vaccine*. 2020;38(22):3862-3868.

Mutsaerts EAML, Nunes MC, Bhikha S, Ikulinda BT, Boyce W, Jose L, Koen A, Moultrie A, Cutland CL, Grobbee DE, Klipstein-Grobusch K, Madhi SA. Immunogenicity and Safety of an Early Measles Vaccination Schedule at 6 and 12 Months of Age in Human Immunodeficiency Virus (HIV)-Unexposed and HIV-Exposed, Uninfected South African Children. *J Infect Dis*. 2019;220(9):1529-38.

Mutsaerts EAML, Nunes MC, van Rijswijk MN, Klipstein-Grobusch K, Grobbee DE, Madhi SA. Safety and Immunogenicity of Measles Vaccination in HIV-Infected and HIV-Exposed Uninfected Children: A Systematic Review and Meta-Analysis. *EClinicalMedicine*. 2018;1:28-42.

Mutsaerts EAML, Nunes MC, van Rijswijk MN, Klipstein-Grobusch K, Otworld K, Cotton MF, Violari A, Madhi SA. Measles Immunity at 4.5 Years of Age Following Vaccination at 9 and 15-18 Months of Age Among Human Immunodeficiency Virus (HIV)-infected, HIV-exposed-uninfected, and HIV-unexposed Children. *Clin Infect Dis*. 2019;69(4):687-696.

Nina Schwalbe, University of the Witwatersrand, 2021-2024.

Thesis: Equitable access to vaccines: exploring the role of the acceptability, accessibility, affordability, availability with a focus on COVID-19.

*Publications from the PhD work:*

Schwalbe N, Nunes MC, Cutland C, Wahl B, Reidpath D. Assessing New York City's COVID-19 Vaccine Rollout Strategy: A Case for Risk-Informed Distribution. J Urban Health. 2024 Apr 5.

Schwalbe N, Hanbali L, Nunes MC, Lehtimaki S. Use of financial incentives to increase adult vaccination coverage: A narrative review of lessons learned from COVID-19 and other adult vaccination efforts. Vaccine X. 2022;12:100225.

Schwalbe N, Nassiri-Ansari T, Lehtimaki S, Cutland C, Nunes MC, Kettler H. Access to medicines: History, Definitions and a Taxonomy of Tools. Submitted.

Tina Ramatsebe, University of the Witwatersrand, 2015 - dissertation submitted in April 2024.

Thesis: Molecular evaluation of the association of the vaginal microbiome and adverse pregnancy outcomes in South Africa.

Sarah Downs, University of the Witwatersrand, 2016 - dissertation submitted in April 2024.

Thesis: Temporal changes in Streptococcus pneumoniae colonization in children and adults following routine childhood immunization with pneumococcal conjugate.

*Publications from the PhD work:*

Olwagen CP, Downs SL, Izu A, Tharasimbi L, Van Der Merwe L, Nunes MC, Madhi SA. Bacterial nasopharyngeal colonisation in children in South Africa before and during the COVID-19 pandemic: an observational study. Lancet Microbe. 2024;5(1):e34-e42.

Downs SL, Olwagen CP, Van Der Merwe L, Nzenze SA, Nunes MC, Madhi SA. Streptococcus pneumoniae and other bacterial nasopharyngeal colonization seven years

post-introduction of 13-valent pneumococcal conjugate vaccine in South African children. *Int J Infect Dis.* 2023;134:45-52.

Downs SL, Madhi SA, Van der Merwe L, Nunes MC, Olwage CP. Optimization of a high-throughput nanofluidic real-time PCR to detect and quantify of 15 bacterial species and 92 *Streptococcus pneumoniae* serotypes. *Sci Rep.* 2023;13(1):4588.

Downs SL, Madhi SA, Van der Merwe L, Nunes MC, Olwage CP. High-throughput nanofluidic real-time PCR to discriminate Pneumococcal Conjugate Vaccine (PCV)-associated serogroups 6, 18, and 22 to serotypes using modified oligonucleotides. *Sci Rep.* 2021;11(1):23728.

William C. McMahon, University of the Witwatersrand, 2021-present.

Thesis: SARS-CoV-2 cell-mediated immunity in South Africans following infection and in COVID-19 vaccinees with and without HIV.

*Publications from the PhD work:*

McMahon WC, Kwatra G, Izu A, Koen AL, Greffrath J, Fairlie L, Patel F, Mukendi CK, Mbele NJ, Lala R, Burgers WA, Nunes MC, Cutland CL, Gilbert SC, Lambe T, Pollard AJ, Madhi SA. T-cell responses induced by ChAdOx1 nCoV-19 (AZD1222) vaccine to wild-type severe acute respiratory syndrome coronavirus 2 among people with and without HIV in South Africa. *AIDS.* 2023;37(1):105-112.

McMahon WC, Kwatra G, Izu A, Jones S, Mbele NJ, Jafta N, Lala R, Shalekoff S, Tiemessen CT, Madhi SA, Nunes MC. T-cell responses to severe acute respiratory syndrome coronavirus 2 ancestral strain and Omicron variant among unvaccinated pregnant and postpartum women living with and without HIV in South Africa. Submitted.

Aung Pone Myint, Université Claude Bernard Lyon1, 2024-present.

Thesis: Phylogenetic dynamics and early variant identification analysis of respiratory viruses genome.

## Masters students

Eleonora Mutsaerts, Utrecht University, the Netherlands, 2015-2016.

Dissertation: Duration of protection provided by trivalent inactivated influenza vaccination in pregnant women.

*Publications from the work:*

Mutsaerts E, Madhi SA, Cutland CL, Jones S, Hugo A, Trenor S, Treurnicht FK, Klipstein-Grobusch K, Weinberg A, Nunes MC. Influenza vaccination of pregnant women protects them over two consecutive influenza seasons in a randomized controlled trial. *Expert Rev Vaccines*. 2016;15(8):1055-62.

Mahlodi Montlha, University of the Witwatersrand, 2015-2017.

Dissertation: Association between cytokine profile and disease severity in children infected with respiratory syncytial virus causing lower respiratory tract infections.

Bronwan Smith, University of the Witwatersrand, 2016-2018.

Dissertation: Determining sequence types of circulating Bordetella pertussis strains isolated from South African infants.

Martijn van Rijswijk, Utrecht University, the Netherlands, 2017.

Dissertation: Long-term follow-up of measles vaccination in HIV-positive children in South Africa.

Noor Gieles, Utrecht University, the Netherlands, 2018-2019.

Thesis: Measles and rubella seroprevalence in HIV-infected and HIV-uninfected pregnant women in Soweto, South Africa.

*Publications from the work:*



Gieles NC, Mutsaerts E, Kwatra G, Bont L, Cutland CL, Jones S, Moultrie A, Madhi SA, Nunes MC. Measles seroprevalence in pregnant women in Soweto, South Africa: a nested cohort study. Clin Microbiol Infect. 2020;26(4):511-515 e514.

Gieles NC, Mutsaerts E, Kwatra G, Bont L, Cutland CL, Jones S, Moultrie A, Madhi SA, Nunes MC. Rubella seroprevalence in pregnant women living with and without HIV in Soweto, South Africa. Int J Infect Dis. 2020;91:255-260.

Natali Serafin, University of Pretoria, South Africa, 2019-2021.

Dissertation: The epidemiology of RSV-associated hospitalisations and risk factors for severe disease among children under 5 years old in Soweto, South Africa, 2014-2019.

Farisai Kuonza, University of the Witwatersrand, 2019-2021.

Dissertation: Hospitalizations for Influenza after introduction of Pneumococcal Conjugate Vaccines at Chris Hani Baragwanath Academic Hospital in South Africa, 2009-2018.

Asha Thombrayil, International Master in Vaccinology (IMVACC), University of Lausanne, Switzerland, 2021-2022.

Dissertation: The Burden of Respiratory Disease Amongst Hospitalized Adults in Soweto, 2017-2019.

Jeanine du Plessis, University of the Witwatersrand, 2021-2022.

Dissertation: Molecular Epidemiology of SARS-CoV-2 in Healthcare Workers at Chris Hani Baragwanath Academic Hospital in Soweto, South Africa.

Publications from the work:

Molecular Epidemiology of SARS-CoV-2 in Healthcare Workers at Chris Hani Baragwanath Academic Hospital in South Africa. In preparation.

## Reviewer of Post-Graduate Dissertations/Theses

PhD of Sibongile Walaza, University of the Witwatersrand, 2018.

Thesis: Interaction between influenza and tuberculosis in South Africa, a country with high prevalence of tuberculosis and HIV.

PhD of Olatunji Oluseyi Adetokunb, Stellenbosch University, South Africa, 2019.

Thesis: Understanding the relationship between vaccination, vaccine-preventable diseases, and HIV infection among sub-Saharan African children.

PhD of Rudzani Muloiwa, University of Cape Town, South Africa, 2020.

Thesis: Epidemiology of pertussis in children hospitalised with respiratory tract infection.

PhD of Patti Sheryl Kay, University of the Witwatersrand, 2021.

Thesis: Phenotypic and functional characterization of CD56bright CD16neg Natural Killer Cell subsets within the context of influenza vaccination.

PhD of Clotilde El Guerche-Séblain, Amsterdam University, the Netherlands, 2022.

Thesis: Strengthening influenza surveillance systems - The contribution of epidemiological and burden of disease data.

PhD of Tumelo Moshoeite, University of the Witwatersrand, 2023.

Thesis: Engineering bispecific antibodies targeting HIV-1 subtype C.

Masters of Rajab Kakaire, University of Cape Town, South Africa, 2017.

Dissertation: The Uptake of the Prepex and Shang ring male circumcision devices by adolescent and adults in Africa: A systematic review.

Masters of Edina Amponsah-Dacosta, University of Cape Town, South Africa, 2019.

Dissertation: Health systems constraints and facilitators of national immunization programs in low- and middle- income countries.

Masters of Linda Erasmus, University of the Witwatersrand, 2019.

Dissertation: Evaluation of case definitions to detect Bordetella Pertussis infections in hospitalised individuals in South Africa, 2015-2017.

Masters of Jessica Ann Lai Yun, University of the Witwatersrand, 2021.

Dissertation: The use of a quantitative assay to differentiate between Pneumocystis jirovecii colonisation and disease among hospitalised patients, South Africa 2015-2016.

Masters of Hannah Theriault, Université Claude Bernard Lyon 1, 2021.

Dissertation: Differential Vulnerability and COVID-19 Vaccination in Sweden: a nationwide ecological study.

## National and International collaborations

| <b>Collaborator's name</b> | <b>Affiliation</b>  | <b>year</b>     |
|----------------------------|---|-----------------|
| Penny Moore                | University of the Witwatersrand   | 2018 to present |
| Cheryl Cohen               | South African, National Institute for Communicable Diseases, South Africa | 2015 to 2023    |
| Michele Ramsay             | University of the Witwatersrand   | 2019 to 2023    |
| Shane Norris               | University of the Witwatersrand   | 2019 to 2022    |
| Ijeoma Edoaka              | University of the Witwatersrand   | 2019 to 2022    |
| Alex Sigal                 | Health Research Institute, South Africa                                   | 2020 to 2022    |
| Keertan Dheda              | University of Cape Town, South Africa                                     | 2020 to 2023    |
| Simon Zwane                | Eswatini Ministry of Health, Eswatini                                     | 2020 to 2023    |
| Celso Khosa                | Universidade Eduardo Mondlane, Mozambique                                 | 2020 to 2023    |
| Louis Bont                 | Utrecht University, the Netherlands                                       | 2017 to present |
| Saverio Caini              | Nivel, the Netherlands  | 2022 to present |
| Francesco Sannino          | Southern Denmark University, Denmark                                      | 2023 to present |
| Francesco Conventi         | Università degli Studi di Napoli, Italy                                   | 2023 to present |
| Gavin Screaton             | University of Oxford, UK  | 2020            |
| Ricardo Aguas              | University of Oxford, UK  | 2020 to 2022    |
| Andrew Pollard             | University of Oxford, UK  | 2020 to 2021    |
| Harish Nair                | University of Edinburgh, Scotland   | 2017 to present |
| Ting Shi                   | University of Edinburgh, Scotland   | 2019 to present |
| Eric Simoes                | Colorado University, USA  | 2012 to present |
| Cecile Viboud              | Fogarty International Center of the National Institutes of Health, USA    | 2023 to present |
| Helen Chu                  | University of Washington, USA   | 2024 to present |
| Saad Omer                  | UT Southwestern Medical Center, USA                                       | 2016 to 2023    |
| Adriana Weinberg           | Colorado University, USA  | 2012 to 2023    |
| Bali Pulendran             | Stanford University, USA  | 2020 to 2022    |
| Barney S. Graham           | National Institute of Allergy and Infectious Diseases, NIH, USA           | 2019 to 2021    |
| Jena Derakshani Hamadani   | icddr,b, Bangladesh   | 2018 to 2023    |

|                      |                          |                 |
|----------------------|--------------------------|-----------------|
| Ana Cumano           | Institut Pasteur, France | 2020 to 2023    |
| Bruno Lina           | UCBL-1, France           | 2017 to present |
| Giacomo Cacciapaglia | UCBL-1, France           | 2023 to present |

## Scientific Journey

### The brain gain

I was one of 16 students selected in 1998 to join the Gulbenkian PhD Program in Biology and Medicine, the most prestigious doctoral program in Portugal at the time. With the option of choosing any scientific laboratory in the world to do my doctoral thesis, I selected the laboratory of Professor Steven Goldman at the Neurology Department of Cornell University, Weill Medical College, New York, NY. During my PhD I isolated a cell population from the white matter of the adult human brain that expressed an immature ganglioside marker. These cells were known to give rise to oligodendrocytes in culture, but my work published in *Nature Medicine* revealed for the first time that in low-density culture some also generated neurons indicating that these nominally glial progenitors included multipotential progenitor cells capable of neurogenesis. I demonstrated that these human progenitor cells could generate functionally competent neurons and glia both in vitro and after xenograft to the fetal rat brain (Marta C. Nunes et al. 2003), and in a dysmyelinated mice model were capable of achieving widespread and efficient myelination (Windrem et al. 2004). These key experiments identified a pool of mitotically competent neurogenic progenitor cells with potential as a therapeutic strategy for a broad range of diseases affecting myelin.

During my PhD, I co-authored four papers (one first author) (Lin et al. 2007; Marta C. Nunes et al. 2007; Windrem et al. 2002; 2004) and one book chapter, and I presented my work at three international conferences.

Below the main paper that emanated from my work.



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[www.nature.com/naturemedicine](http://www.nature.com/naturemedicine)

## Brain gain

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Answers from the A $\beta$  vaccine trial

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On osteopetrosis: identification of grey-lethal

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Metastasis signature for liver cancer

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TIMP3: a new ligand for VEGFR2

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Retroviral transgene safety

# Identification and isolation of multipotential neural progenitor cells from the subcortical white matter of the adult human brain

MARTA C. NUNES<sup>1</sup>, NEETA SINGH ROY<sup>1</sup>, H. MICHAEL KEYOUNG<sup>1</sup>, ROBERT R. GOODMAN<sup>2</sup>, GUY MCKHANN II<sup>2</sup>, LI JIANG<sup>3</sup>, JIAN KANG<sup>3</sup>, MAIKEN NEDERGAARD<sup>3</sup> & STEVEN A. GOLDMAN<sup>1</sup>

<sup>1</sup>Department of Neurology and Neuroscience, Cornell University Medical College, New York, New York, USA

<sup>2</sup>Department of Neurosurgery, Columbia University College of Physicians and Surgeons, New York, New York, USA

<sup>3</sup>Department of Anatomy and Cell Biology, New York Medical College, Valhalla, New York, USA

M.C.N. and N.S.R. contributed equally to this work.

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**The subcortical white matter of the adult human brain harbors a pool of glial progenitor cells. These cells can be isolated by fluorescence-activated cell sorting (FACS) after either transfection with green fluorescent protein (GFP) under the control of the *CNP2* promoter, or A2B5-targeted immunotagging. Although these cells give rise largely to oligodendrocytes, in low-density culture we observed that some also generated neurons. We thus asked whether these nominally glial progenitors might include multipotential progenitor cells capable of neurogenesis. We found that adult human white-matter progenitor cells (WMPCs) could be passaged as neurospheres *in vitro* and that these cells generated functionally competent neurons and glia both *in vitro* and after xenograft to the fetal rat brain. WMPCs were able to produce neurons after their initial isolation and did not require *in vitro* expansion or reprogramming to do so. These experiments indicate that an abundant pool of mitotically competent neurogenic progenitor cells resides in the adult human white matter.**

The adult human subcortical white matter harbors a population of mitotically competent glial progenitors that comprise as many as 3% of its cells<sup>1,2</sup>. These cells may be extracted from brain tissue using FACS after transfection with GFP-encoding plasmids driven by the promoter for *CNP*, an early oligodendrocytic transcript<sup>2,3</sup>. The cells express the immature neural ganglioside recognized by monoclonal antibody A2B5 but do not express more mature markers of glial lineage. We previously noted that when grown at high density, pCNP2:hGFP<sup>+</sup> progenitors gave rise to glia, largely oligodendrocytes. Nonetheless, in low-density culture after high-purity FACS, pCNP2:hGFP<sup>+</sup> cells often generated  $\beta$ III-tubulin<sup>+</sup> neurons<sup>2</sup>. Because neurogenesis was never observed from pCNP2:hGFP<sup>+</sup> cells in higher-density or unsorted cultures, we postulated that the restriction of these progenitor cells to the oligodendroglial phenotype might be an effect of environmental cues rather than a function of autonomous commitment. Once isolated into high-purity, low-density culture, and therefore removed from any paracrine or autocrine influences, human subcortical pCNP2:hGFP<sup>+</sup> cells were able to generate neurons as well as glia<sup>2</sup>. It was subsequently reported<sup>4</sup> that glial progenitors from the postnatal rat optic nerve could also generate neurons after serum- or bone morphogenetic protein-induced phenotypic instruction and basic fibroblast growth factor (bFGF)-stimulated expansion. Similar work showed that progenitor cells of the adult rat forebrain parenchyma could also generate neurons after prolonged *in vitro* expansion in bFGF<sup>8</sup>. Taken to-

gether, these findings indicated that glial progenitor cells might retain substantial phenotypic plasticity.

We asked whether some fraction of the nominally glial progenitors of the adult human subcortical white matter might actually be parenchymal neural stem cells. Specifically, we asked whether single, sorted WMPCs could generate multiple neural phenotypes, and if so, whether they were capable of expansion and self-renewal. In addition, we investigated whether this process requires de-differentiative reprogramming to an intermediate phenotype, or whether simply removing these cells from their local environment and mitotically expanding them in bFGF might suffice to permit these cells to act as multipotential progenitors. In doing so, we tested the hypothesis that the phenotypic plasticity of adult WMPCs might be tonically restricted by the adult parenchymal environment, rather than irreversibly lost with development.

## WMPCs were isolated by *CNP*- and A2B5-based sorting

White matter was dissected from surgical samples taken at the time of temporal lobectomy for epilepsy, aneurysm, and post-traumatic decompression ( $n = 21$ ). The tissues were dissected free of adjacent cortex and ventricular epithelium, and enzymatically dissociated to single-cell suspension as described<sup>2</sup>. The dissociates were plated onto laminin (100  $\mu$ g/ml) in DMEM/F12/N1 supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and platelet-derived growth factor (PDGF)-AA (20 ng/ml). To identify oligodendrocyte progenitors, the dissociates were trans-



ected with pCNP2:hGFP, the transcription of which results in GFP expression by oligodendrocyte progenitor cells<sup>2</sup>.

To avoid both the temporal lag between transfection and GFP expression and the inefficiency of plasmid transfection, cultures were also sorted on the basis of A2B5 surface immunoreactivity, which can serve as a surrogate marker for pCNP2:hGFP<sup>+</sup> WMPCs *in vitro*<sup>2</sup>. Immunostaining showed that 84 ± 8.3% of pCNP2:GFP<sup>+</sup> cells expressed A2B5 (ref. 2). GFP-based FACS gated 0.49 ± 0.15% of all white-matter cells as pCNP2:hGFP<sup>+</sup> (mean ± s.e.m.; *n* = 3 patients; Fig. 1a). Matched cultures transfected with pCMV:GFP had a net transfection efficiency of 13.1%. Thus, the predicted incidence of pCNP2:hGFP<sup>+</sup> cells in the white matter was 3.7% (= 1 + 0.131 × 0.49), consistent with our prior estimates of the incidence of this phenotype<sup>2</sup>. From the same samples, A2B5-based FACS gated an average of 3.1 ± 0.7% (*n* = 3) of the white-matter cell population (Fig. 1b). The greater than six-fold increase in net yield when A2B5 was used (3.1% versus 0.49%) reflected the higher efficiency of A2B5 immunodetection relative to pCNP2:hGFP plasmid transfection. On this basis, we used immunomag-

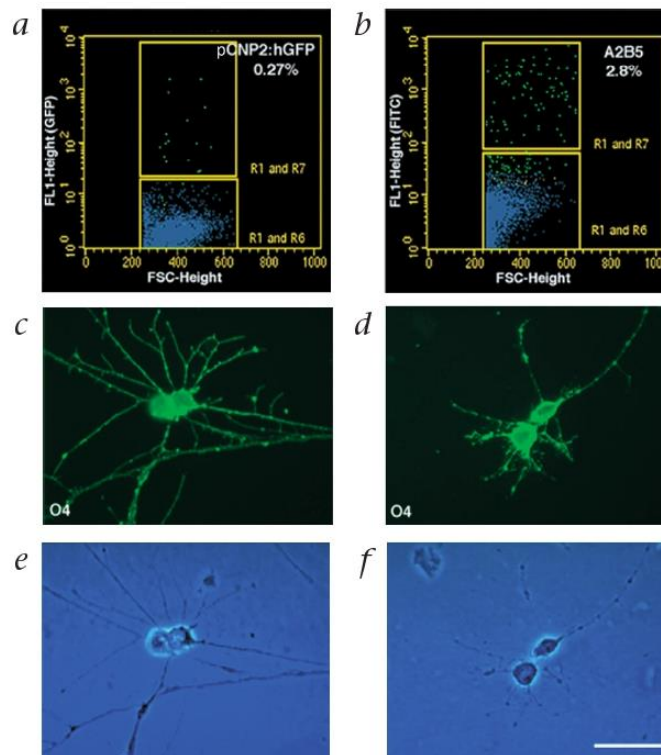
netic sorting (IMS) to select A2B5<sup>+</sup> cells from adult white-matter dissociates. By IMS, the incidence of A2B5-sorted cells in white matter dissociates was 3.6 ± 0.3% (*n* = 21) with a median of 3.1%. This improved yield was accomplished with no appreciable loss of cell-type specificity, in that the A2B5<sup>+</sup> cells overlapped entirely with the sort profiles of pCNP2:hGFP<sup>+</sup> cells and each isolate generated O4<sup>+</sup> oligodendrocytes with similar efficiency (Fig. 1c–f). Thus, A2B5-based FACS and IMS identified WMPCs homologous to those recognized by pCNP2:GFP-based FACS, while permitting higher-yield isolation of these cells.

#### Adult WMPCs gave rise to multipotent neurospheres

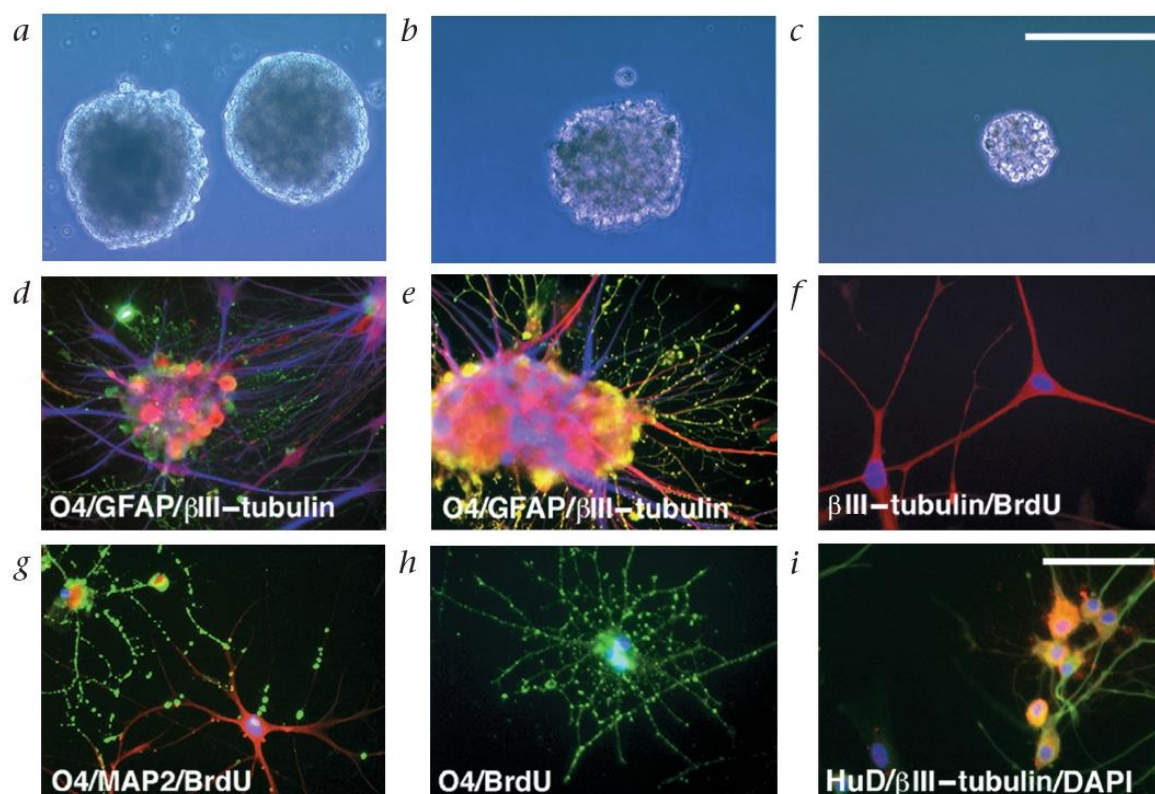
To assess the expansion capacity of pCNP2:hGFP<sup>+</sup> and A2B5-sorted cells, we propagated sorted isolates of each in suspension<sup>6–8</sup>. The cells were distributed into 24-well plates at 50,000 cells per 0.5 ml in serum-free media (SFM) supplemented with bFGF (20 ng/ml), NT3 (2 ng/ml) and PDGF-AA (20 ng/ml), a combination that permits the expansion of human WMPCs<sup>2</sup>. Seven days later, the cells were switched to SFM with bFGF alone (20 ng/ml)<sup>8</sup>. Over the next 10 d, neurospheres—spherical masses

of cells that expand from single parental progenitors—arose in these cultures, such that by 3 weeks after sorting, there were 84.8 ± 9.0 spheres/well (*n* = 4 patients). These neurospheres were typically >150 μm in diameter and included 46.5 ± 8.2 cells/sphere (Fig. 2a and b). Thus, single WMPCs of the adult human brain were capable of generating neurospheres.

To establish the lineage potential of single adult human WMPCs, we dissociated the resultant primary neurospheres and passaged them into new wells. Alternatively, some were plated onto substrate to permit their differentiation. Immunostaining showed that both pCNP2:hGFP<sup>+</sup> and A2B5<sup>+</sup> progenitor-derived spheres gave rise to all major neural phenotypes (Fig. 2d and e). Among those cells passaged from primary spheres, secondary spheres were observed to arise within two weeks after passage. After expansion, these secondary spheres were similarly plated on substrate, raised for one to two weeks and fixed. Immunolabeling confirmed that virtually all secondary spheres generated both neurons and glia together (Fig. 2c and e). In addition, when the mitotic marker BrdU was added to A2B5-sorted cells, BrdU-incorporating neurons, oligodendrocytes and astrocytes all emerged from the spheres generated (Fig. 2f–i). The persistence of mitotic neurogenesis and gliogenesis by single spheres indicated that they contained cycling multipotential cells. The secondary spheres were probably of clonal origin, given the low plating density of the single cells from which each was derived and the fact that the sphere-forming cells originated from primary spheres that had themselves expanded from single-cell dissociates. These data indicate that single progenitor cells of the adult human white matter are both clonogenic and multipotent.



**Fig. 1** A2B5-based FACS selects oligodendrocyte progenitor cells. **a** and **b**, FACS graphs showing the extraction of pCNP2:hGFP<sup>+</sup> (**a**) and A2B5<sup>+</sup> (**b**) WMPCs from an adult human white-matter dissociate. Forward scatter (FCS), a measure of cell size, is plotted against fluorescence intensity (FL-1). When pCNP2:hGFP- and A2B5-based sorts were directly compared, their plots showed overlapping profiles, but A2B5<sup>+</sup> cells were >6-fold more abundant than their pCNP2:hGFP<sup>+</sup> counterparts, reflecting the higher efficiency of A2B5 surface tagging. **c–f**, Progenitors sorted by pCNP2:hGFP (**c** and **e**) and A2B5 (**d** and **f**) gave rise to O4<sup>+</sup> oligodendrocytes. A2B5-based surface antigen sorting may thus be used as a higher-yield alternative to pCNP2:hGFP transfection-based FACS for isolating WMPCs. Scale bar, 24 μm.



**Fig. 2** Adult human WMPCs give rise to multipotential neurospheres. **a**, First-passage spheres generated from A2B5-sorted cells 2 weeks after sorting. **b**, First-passage spheres arising from pCNP2:hGFP-sorted cells, at 2 weeks. **c**, Second-passage sphere derived from an A2B5-sorted sample, at 3 weeks. **d**, Once plated onto substrate, the primary spheres differentiated into  $\beta$ III-tubulin<sup>+</sup> neurons (red), GFAP<sup>+</sup> astrocytes (blue) and O4<sup>+</sup> oligodendrocytes (green). **e**, Neurons (red), astrocytes (blue) and oligodendrocytes (green) arose similarly from spheres derived from pCNP2:GFP-sorted WMPCs. **f–h**, BrdU incorporation (blue) showed that new neurons (**f**,  $\beta$ III-tubulin (red); **g**, MAP2 (red)) and oligodendrocytes (**h**, O4 (green)) were generated *in vitro*. **i**,  $\beta$ III-tubulin<sup>+</sup> neurons (green) co-expressed neuronal Hu protein<sup>40,41</sup> (red), yielding double labeling (yellow). Nuclei were counterstained with DAPI (blue). Scale bars, 100  $\mu$ m (**a–e**) or 40  $\mu$ m (**f–i**).

### Single WMPCs remained multipotential with passage

The serial propagability of sorted WMPCs from neurospheres in low-density dissociates suggested the clonal derivation of each individual sphere<sup>9–11</sup>. To further validate the clonal origin of neurons and glia arising within single spheres, we used lentiviral GFP to genetically tag and follow single WMPCs. A2B5<sup>+</sup> cells were tagged, 2–5 d after sorting, with a lentivirus expressing GFP under cytomegalovirus (CMV) promoter control<sup>12–14</sup>. At 10 PFU/cell, 23% of the cells expressed GFP by one week after sorting, yielding a mixture of GFP<sup>-</sup>, GFP<sup>+</sup> and mixed spheres in the resultant cultures (Fig. 3a–b). These primary spheres were triturated two to four weeks later to single-cell suspensions and passaged into bFGF at ~3,000 cells/well. Under these conditions,  $40.8 \pm 12.9$  secondary spheres/well were generated, indicating a clonogenic cell incidence of 1.3% ( $n = 5$ ). Of these secondary spheres,  $47.2 \pm 10.8\%$  contained only GFP<sup>+</sup> cells (Fig. 3c–d) whereas  $30.9 \pm 6.9\%$  harbored no GFP<sup>+</sup> cells. The relative uniformity of GFP expression, or lack thereof, among the cells within a given sphere indicated that most spheres were clonally derived ( $P < 0.005$  by  $\chi^2$  analysis). This tested the null hypothesis that the spheres arose from non-clonal aggregation of two or more cells, each of which was

equally likely to be GFP<sup>+</sup> or GFP<sup>-</sup>. When the single spheres were plated onto polyornithine and fibronectin and their outgrowth assessed two weeks later, all gave rise to both neurons and glia (Fig. 3e–g). Because most secondary spheres were likely to have been clonally derived, and all included neurons as well as glia (38 of 38 spheres;  $n = 4$  samples), single WMPCs must have given rise to neurons and glia together.

We next asked if the neurogenic capacity and multilineage competence of WMPCs were maintained with passage. Primary spheres were raised serially in bFGF/NT3/PDGF-AA for 7 d, DMEM/F12/N1 with 15% serum/PDGF-AA for 4 d, and serum-free DMEM/F12/N1 with bFGF for 10 d. Cells were then dissociated and replated in bFGF at 3,000 cells/well in a 24-well plate. Secondary spheres arose within two weeks from  $1.1 \pm 0.3\%$  of these cells ( $n = 8$ ). After more than two weeks of further expansion, the secondary spheres were plated on polyornithine and fibronectin and were fixed and immunostained two weeks later (seven to nine weeks after sorting). Whereas primary spheres consisted of  $21.7 \pm 4.3\%$   $\beta$ III-tubulin<sup>+</sup> neurons,  $17.7 \pm 3.9\%$  glial fibrillary acidic protein (GFAP)<sup>+</sup> astrocytes and  $46.7 \pm 5.9\%$  O4<sup>+</sup> oligodendrocytes ( $n = 3$ ), secondary spheres consisted of  $16.0 \pm 2.5\%$  neurons,  $19.3 \pm 3.2\%$  astro-

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cytes and  $46.4 \pm 2.4\%$  oligodendrocytes ( $n = 3$ ). Most of the neurons were GABAergic, by virtue of their expression of glutamic acid decarboxylase-67 (GAD67) (Fig. 4a–c). Because the relative proportions of neurons, oligodendrocytes and astrocytes in secondary spheres were similar to those in primary spheres, we concluded that WMPCs retained multilineage competence with expansion.

### WMPC-derived neurons become functionally mature

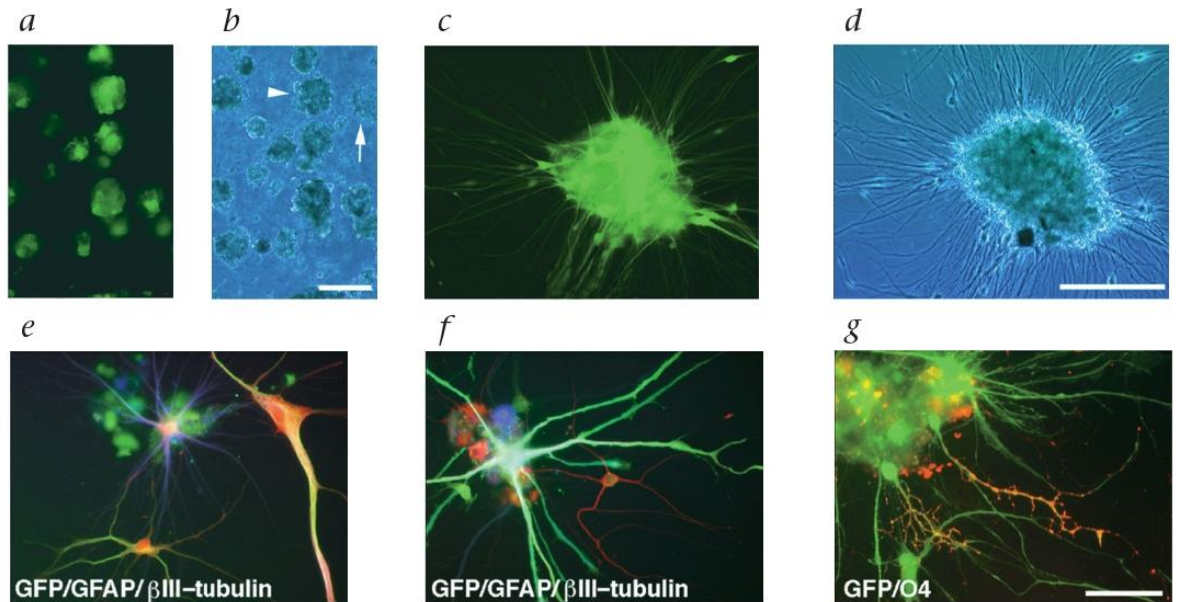
The calcium responses and membrane currents of WMPC-derived neurons were assessed to establish their ability to respond to depolarizing stimuli. Primary spheres ( $n = 12$  fields, derived from 3 brains) were plated on fibronectin to permit neuronal outgrowth, and assessed 14 d later for their calcium responses to depolarizing stimuli. The cultures were then loaded with the calcium indicator dye Fluo-3 and serially exposed to both 100  $\mu\text{M}$  glutamate and 60 mM potassium during confocal microscopy. Astrocytic responses to depolarization were minimal under these culture conditions, as previously noted. In contrast, neuron-like cells displayed rapid, reversible, >100% elevations in cytosolic calcium in response to potassium, consistent with the activity of neuronal voltage-gated calcium channels (Fig. 4d–f). The neuronal phenotype of these cells was then validated by immunostaining for  $\beta$ III-tubulin.

We then asked whether WMPC-derived neurons would be able to develop the fast sodium currents and action potentials characteristic of electrophysiologically competent neurons. We used whole-cell patch-clamp recording during current stimulation to assess the response of WMPC-derived neurons that arose from plated secondary spheres derived from A2B5-

sorted isolates. A total of 58 WMPC-derived fiber-bearing cells were recorded, in 5 cultures derived from 3 patients. Of these, 13 showed voltage-activated sodium ion currents ( $I_{\text{Na}}$ ) of >100 nA, and 7 had  $I_{\text{Na}} > 600$ , compatible with the fast sodium currents of neuronal depolarization<sup>15,16</sup>. Accordingly, whereas two of five cells with  $I_{\text{Na}} > 800$  generated stimulus-evoked action potentials (Fig. 4g–h), none did so with  $I_{\text{Na}} < 800$ . In addition, none of 26 morphologically non-neuronal cells showed substantial ( $\geq 100$  pA) current-induced sodium currents. Together, these results indicated that neurons arising from adult human WMPCs developed mature electrophysiologic functions, including both fast sodium currents and action potentials.

### WMPCs generated neurons without reprogramming

Glial progenitor cells from the postnatal rat optic nerve can generate neurons, under conditions that have been described as ‘reprogramming’ glial progenitors to multilineage competence<sup>4</sup>. In that study, neurogenesis was achieved by first structuring the cells to an intermediary astrocytic lineage using either serum or bone morphogenetic protein-2, followed by bFGF-stimulated mitogenesis. We asked whether such reprogramming steps are required for the generation of neurons from adult human WMPCs, or whether simple expansion under minimal conditions *in vitro*, with the removal of these cells from their environment, might be sufficient to permit neurogenesis by these cells. Sorted A2B5<sup>+</sup> cells were cultured in several permutations of mitogenic and differentiative conditions to identify the minimal conditions permissive for lineage diversification. We compared the phenotypes generated under three conditions: (i) bFGF/NT3/PDGF-AA in SFM (composed of DMEM/F12/N1) for 7 d, followed by 15% FBS/PDGF-



**Fig. 3** Single lentiviral GFP-tagged WMPCs generated neurons and glia. A2B5-sorted WMPCs were infected with a lentivirus encoding enhanced GFP<sup>14</sup>, 5 d after sorting. **a** and **b**, Secondary spheres subsequently derived from infected cells harbored either GFP-tagged cells (arrowhead), untagged cells (arrow) or, less commonly, both. **c** and

**d**, GFP<sup>+</sup> secondary sphere 1 week after plating. **e** and **f**,  $\beta$ III-tubulin<sup>+</sup> neurons (red) and GFAP<sup>+</sup> astrocytes (blue) arising from a single clonally derived GFP<sup>+</sup> secondary sphere. **g**, GFP<sup>+</sup> (green) and O4<sup>+</sup> (red) oligodendrocytes arising from a secondary sphere. Scale bars, 100  $\mu\text{m}$  (a and b), 60  $\mu\text{m}$  (c and d) or 40  $\mu\text{m}$  (e–g).

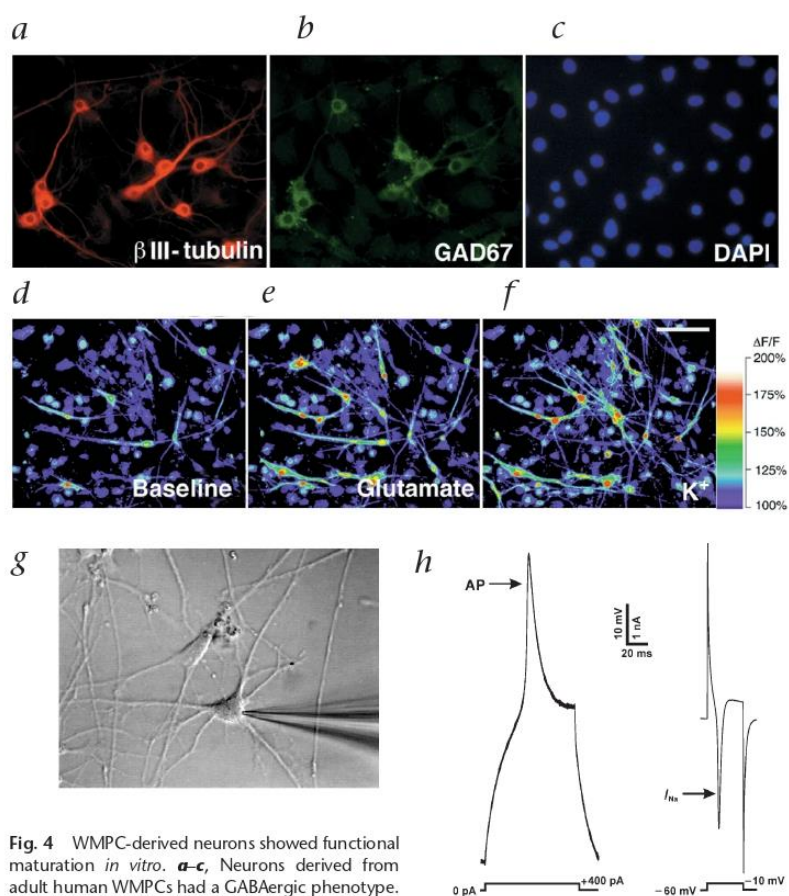
AA for 4 d and SFM with bFGF for two weeks; (ii) bFGF/NT3/PDGF-AA in SFM continuously for three weeks; and (iii) bFGF alone in SFM for three weeks. The first condition was intended to promote initial differentiation in serum, whereas the latter two groups were designed to skip this glial differentiative step<sup>4</sup>.

The A2B5-sorted progenitors yielded spheres under each of these conditions; however, both the number of spheres and the percentage of neurons generated by each differed as a function of treatment. Cultures maintained in base media alone or in bFGF-supplemented media had  $5.9 \pm 1.7\%$  and  $7.2 \pm 2.1\%$   $\beta$ III-tubulin<sup>+</sup> neurons, respectively ( $n = 3$  patients). When matched WMPC-derived spheres were sequentially raised in bFGF/NT3/PDGF-AA with 15% serum and bFGF,  $18.2 \pm 2.2\%$  of the cells were  $\beta$ III-tubulin<sup>+</sup> (Fig. 5a). A similar proportion of neurons ( $22.5 \pm 1.9\%$ ;  $n = 3$ ) was generated by those neurospheres maintained in SFM with bFGF/NT3/PDGF-AA. Serum exposure was therefore not required for A2B5<sup>+</sup> cells to generate neurons. Indeed, no specific signals seemed necessary for neuronal instruction, besides those provided by PDGF and NT3. These data indicated that antecedent astrocytic differentiation was not a necessary prerequisite to neurogenesis by adult WMPCs. These cells required neither prolonged mitogenic expansion, nor specific dedifferentiation steps, to generate neurons as well as glia<sup>4</sup>.

Although both PDGF and NT3 promote oligodendrocyte production by glial progenitors of the rat optic nerve<sup>17,18</sup>, each can induce neuronal differentiation in less-committed hippocampal and ventricular zone neural progenitors<sup>19,20</sup>. As such, their neurogenic effects on adult WMPCs may reflect the relatively undifferentiated state of these cells.

#### Only a fraction of A2B5<sup>+</sup> cells were clonogenic

We next assessed the incidence of clonogenic and multipotential progenitor cells within the larger pool of A2B5-sorted white-matter cells. We first assessed whether either the survival or the mitotic competence of adult human WMPCs were dependent on density, by assessing the limiting dilution at which clonogenic progenitors could be obtained from A2B5-sorted white-matter dissociates. A2B5<sup>+</sup> cells were plated immediately after sorting, at densities ranging from 100,000 to 1,000 cells/ml (0.5 ml cell suspension per well of a 24-well plate), in basal media supple-



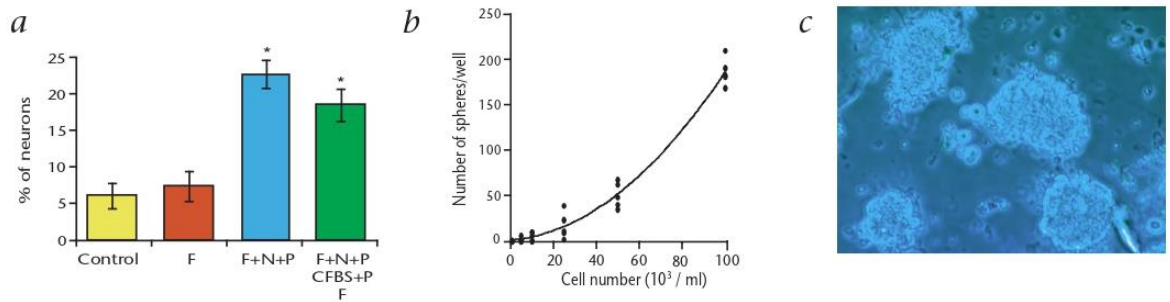
**Fig. 4** WMPC-derived neurons showed functional maturation *in vitro*. **a–c**, Neurons derived from adult human WMPCs had a GABAergic phenotype. **a**, Outgrowth of a WMPC-derived neurosphere, stained for neuronal  $\beta$ III-tubulin after 35 d *in vitro*.

**b**, Immunostaining showed that all 9 neurons in the field were GAD67<sup>+</sup> and were thus likely to be GABAergic. **c**, DAPI nuclear counterstaining showed the abundance of cells in the field. **d–f**, WMPC-derived neurons developed neuronal Ca<sup>2+</sup> responses to depolarization. **d**, WMPC-derived cells loaded with the calcium indicator dye Fluo-3, 10 d after plating of first-passage spheres derived from A2B5-sorted white matter (35 d *in vitro* total). Many fiber-bearing cells of both neuronal and glial morphologies are apparent. **e**, The same field after exposure to 100  $\mu$ M glutamate. **f**, The same field after exposure to a depolarizing stimulus of 60 mM KCl. Rapid, reversible, >100% elevations in cytosolic calcium occurred in response to K<sup>+</sup>, consistent with the activity of neuronal voltage-gated calcium channels. Scale bar, 80  $\mu$ m. **g** and **h**, Whole-cell patch-clamp experiments detected voltage-gated sodium currents and action potentials in WMPC-derived neurons. **g**, Representative cell, 14 d after plating of first-passage sphere derived from A2B5-sorted white matter. The cell was patch clamped in a voltage-clamped configuration and its responses to current injection were recorded. **h**, Action potentials (AP) were noted after positive current injection, at  $I_{inj} > 800$  pA (left tracing). The fast negative deflections noted after depolarization steps are typical of the voltage-gated sodium currents of mature neurons (right).

mented with bFGF/NT-3/PDGF-AA. Under these conditions, the incidence of clonogenic progenitors was a curvilinear function of the sorted cell density ( $R^2 = 0.978$ ; Fig. 5b). Whereas 186  $\pm$  7.6 spheres were generated at a density of 100,000 cells/ml (0.4%;  $n = 5$  patients), only 6.5  $\pm$  2.7 were noted at 10,000 cells/ml (0.1%) and no sphere generation was noted at or below 5,000 cells/ml. Thus, the expansion of purified WMPCs was density dependent and optimal at 50,000–100,000 cells/ml. Densities higher than the optimal range seemed to promote terminal differentiation of the progenitors.



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**Fig. 5** WMPCs show density-dependent expansion and neurogenesis. **a**, WMPCs can generate neurons after initial isolation. When A2B5-sorted cells were maintained in base medium alone or in bFGF-supplemented medium, 5–7% βIII-tubulin/Tuj1<sup>+</sup> neurons were observed in the culture. WMPC-derived spheres raised continuously in bFGF/NT3/PDGF-AA or sequentially in bFGF/NT3/PDGF-AA, 15% serum/PDGF-AA and bFGF, gave rise to progressively higher percentages of neurons (see text). \*,  $P < 0.01$  by one-way analysis of variance with Bonferroni *t*-test. F, bFGF; N, NT3; P, PDGF-AA; CFBS, characterized fetal bovine serum. **b**, Adult human WMPCs show density-dependent expansion, such that no sphere formation was observed below a cell density of 10,000 cells/ml. The incidence of sphere formation was a curvilinear function of cell density ( $R^2 = 0.9781$ ). **c** and **d**, Only A2B5-selected cells generated spheres. **c**, First-passage spheres generated from A2B5<sup>+</sup> cells 2 weeks after sorting. **d**, A2B5-depleted remainder of A2B5<sup>+</sup>-cells, derived from the same source culture as cells in (c), exhibited no evidence of sphere formation 2 weeks after sorting.

To assess whether clonogenic WMPCs were restricted to the A2B5<sup>+</sup> population, we also cultured the A2B5-depleted pool remaining after each sort. A2B5-depleted cultures did not give rise to any passageable neurospheres at any of the cell densities assessed over the range of 1,000–100,000 cells/ml (Fig. 5d). On the basis of these studies, we concluded that only a fraction of white-matter A2B5<sup>+</sup> cells are actually clonogenic and multipotential progenitors, although all clonogenic WMPCs are A2B5<sup>+</sup>.

**Adult WMPCs showed limited self-renewal**

We next sought to define the extent to which WMPCs were self-renewing by assessing the extent to which WMPC-derived neurospheres were capable of repetitive passage. Primary spheres were raised from three patients at an optimal initial density of 100,000 cells/ml, under the conditions identified as most supportive of multilineage expansion (bFGF/NT3/PDGF-AA in DMEM/F12/N1). One month later, the spheres were dissociated and replated. Secondary spheres were generated and were replated one month later at  $1 \times 10^4$ – $5 \times 10^4$  cells/ml. These cultures gave rise to tertiary spheres over the following month, though with less efficiency and a smaller volumetric expansion than secondary spheres. Attempts at propagating these spheres as quaternary spheres, after additional dissociation, were generally unsuccessful. Given an apparent cell doubling time of 3–4 d (data not shown) and monthly passages spanning 8–10 doublings, we estimated that the tertiary spheres assessed one month after the last passage underwent a minimum of 16–24 and no more than 30 doublings. This is well below the number of doublings of which tissue-derived stem cells are typically thought capable.

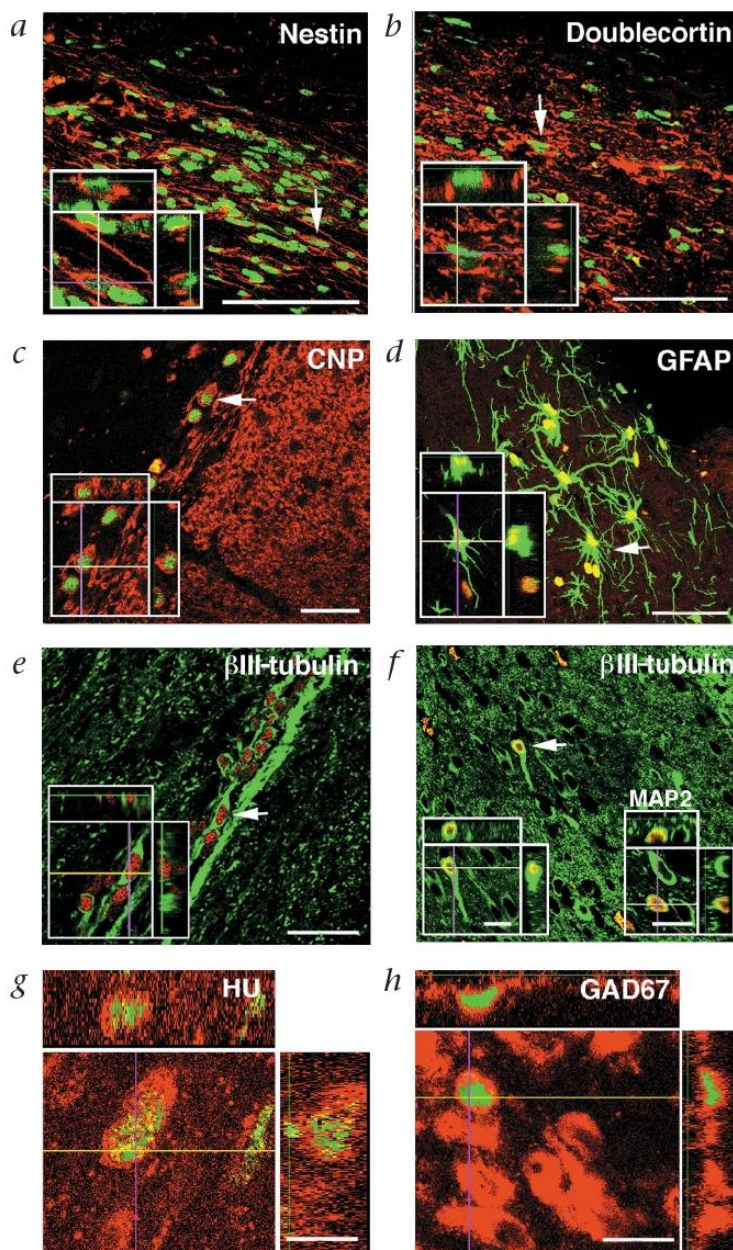
Our inability to successfully passage these cells beyond 16–24 doublings called into question their ability to self-replicate for extended periods of time *in vitro*. Their limited replicative competence contrasted with that of neural progenitors sorted from the fetal human ventricular zone, which may be readily passaged for >60 doublings under analogous culture conditions<sup>21</sup>.

Such self-renewal capacity has been ascribed to sustained telomerase activity in a number of developing systems, including the fetal human forebrain<sup>22,23</sup>. To assess whether the apparently finite proliferative potential of adult human WMPCs reflected a lack of telomerase activity, telomerase levels were assessed using the telomerase reverse transcriptase activity protocol (TRAP) assay<sup>23,24</sup>. We did not detect any telomerase activity in primary or secondary WMPC-derived spheres, despite high-level activity in a variety of positive controls (see Supplementary Fig. 1 online). Their lack of extended replicative potential, coupled with their lack of telomerase activity, suggests that adult WMPCs might constitute a pool of multipotential progenitors with a finite capacity for mitotic expansion, transitional between tissue-restricted stem cells and phenotypically committed progenitors.

**WMPCs produced neurons and glia after fetal xenograft**

We next assessed whether WMPCs were multipotential *in vivo* as well as *in vitro* by evaluating their fate after engraftment to embryonic stage (E)17 fetal rat brains. Some A2B5-sorted cells were transplanted 24–48 h after sorting to assess their lineage potential upon initial isolation. These cells were maintained only in SFM during the period between isolation and xenograft and were never exposed to any exogenous growth factors. Other cells were transplanted 10 d after sorting, after maintenance in bFGF/NT3/PDGF-AA for 4 d and 15% serum/PDGF-AA followed by bFGF, for 3 days each. All donor cells were administered into E17 rat embryos by intraventricular injection at  $10^5$  cells/animal. The recipients were killed and fixed four weeks after birth to evaluate the fate of the implanted human cells. Human donor cells were identified by immunolabeling of brain sections for human nuclear antigen (HNA).

In rats implanted with propagated WMPCs (Fig. 6) and their counterparts injected with acutely isolated WMPCs (see Supplementary Fig. 2 online), donor-derived migrants co-expressing HNA with either nestin or doublecortin<sup>25</sup> were found in the host olfactory subependyma and hippocampus (Fig. 6a and



**Fig. 6** WMPCs engrafted into fetal rats give rise to neurons and glia in a site-specific manner. Sections from a rat brain implanted at E17 with A2B5-sorted WMPCs and killed 1 month after birth. Cells were maintained in culture for 10 d before implanting. **a** and **b**, Nestin<sup>+</sup> (**a**) progenitors and doublecortin<sup>+</sup> (**b**) migrants (red) each co-expressing HNA (green) in the hippocampal alvius. **c**, CNP<sup>+</sup> (red) HNA<sup>+</sup> (green) oligodendrocytes, found exclusively in the corpus callosum. **d**, Low-power image of GFAP<sup>+</sup> (green) HNA<sup>+</sup> (red) astrocytes (yellow, double-positive) along the ventricular wall. **e**,  $\beta$ III-tubulin<sup>+</sup> (green) and HNA<sup>+</sup> (red) neurons migrating in a chain in the hippocampal alvius. **f**,  $\beta$ III-tubulin<sup>+</sup> and MAP2<sup>+</sup> (inset) neurons in the striatum, adjacent to the rostral migratory stream (green,  $\beta$ III-tubulin and MAP2; red, HNA; yellow, double-stained human nuclei). **g**, Hu<sup>+</sup> (red) HNA<sup>+</sup> (green) neuron in the septum. **h**, GAD67<sup>+</sup> (red) HNA<sup>+</sup> (green) striatal neuron. Insets (**a–f**) show orthogonal projections of a high-power confocal image of each identified cell (arrow). Scale bars, 40  $\mu$ m (**a–e**) or 20  $\mu$ m (**f–h**).

**b**). In addition, abundant populations of HNA<sup>+</sup>  $\beta$ III-tubulin<sup>+</sup> neurons were found in the olfactory subependyma and rostral migratory stream as well as in the hippocampal alvius (Fig. 6e). WMPC-derived neurons were also observed in the neostriatum, indicating striatal neuronal differentiation on the part of some xenografted WMPCs (Fig. 6f). These data showed that engrafted adult human WMPCs could integrate into the forebrain subventricular zone as neuronal progenitor cells that then gave rise to both granule and striatal neurons. Human WMPC-derived GFAP<sup>+</sup> astrocytes and CNP<sup>+</sup> oligodendrocytes were also common in recipient brains and were found primarily along the ventricles or in the subcortical white matter (Fig. 6c and d). Thus, adult human WMPCs showed context-dependent differentiation after xenograft to the developing rat brain and were competent to do so upon acute isolation, without the benefit of humoral instruction *in vitro*.

#### Discussion

These observations suggest that the WMPCs of the adult human forebrain include multipotential progenitor cells, capable of a finite and limited degree of expansion and self-renewal. These cells remain competent to respond to local instructive cues, with a wide range of lineage choices, upon xenograft as well as *in vitro*. They are readily able to give rise to neurons and glia once they are removed from their native white-matter environment. The freshly isolated adult WMPCs in our study did not require prolonged expansion to undergo neurogenesis *in vitro*, and seemed immediately competent to generate neurons upon xenograft to the developing brain.

Previous studies of the adult rat brain have identified parenchymal progenitor cells that are able to give rise to neurons and glia after a number of cell doublings, in the presence of bFGF<sup>8</sup>. In addition, nominally committed glial progenitor cells derived from the neonatal rat optic nerve have also been reported to give rise to neurons and oligodendrocytes<sup>4</sup>. The lineage diversification of these cells seems to require a humorally directed reprogramming of their phenotype, with the induction of an astrocytic intermediary on the way to neurogenesis. In the present study, adult human WMPCs did not seem to require any such reprogramming or transdifferentiation to achieve multilineage competence. Similarly, they did not seem to pass through an intermediate astrocytic stage before generating neurons, oligodendrocytes and astrocytes. Indeed, after their acute isolation and xenograft, A2B5-defined WMPCs were able to

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generate all major neural phenotypes *in vivo* and *in vitro*, without any exogenous growth factor exposure. Nevertheless, because an average of 7% of A2B5-sorted white-matter cells co-expressed GFAP (data not shown), it is possible that some WMPCs exhibit astroglial features at some point during their ontogeny, much like subventricular neural progenitor cells<sup>26,27</sup>. This categorization notwithstanding, our results suggest that the WMPCs of the adult human brain are fundamentally tissue-specific progenitor cells that are tonically restricted to glial lineage by the local parenchymal environment, and do not require specific phenotypic reprogramming for neuronal differentiation.

These data suggest that adult human WMPCs constitute a population of parenchymal glial progenitor cells whose *in situ* fate is restricted by the local white-matter environment. Yet the progenitor cell pool of the adult white matter may be heterogeneous, and it is not clear whether all WMPCs have the same ontogeny or fate potential<sup>28–30</sup>. A minority of multipotential progenitor cells might still persist among a larger pool of more fundamentally lineage-restricted glial progenitors<sup>9</sup>. These parenchymal multipotent progenitors may constitute a relatively rare subpopulation, more akin to persistent stem cells than to any lineage-restricted derivatives<sup>31,32</sup>. In this regard, although we did not detect telomerase activity in sorted WMPCs, if the clonogenic portion of these represents only a small fraction of the total progenitor pool, then their numbers might have been below the detection threshold of our TRAP assay. Further study of the heterogeneity of the white-matter progenitor cell population, and of the lineage competence of its constituent phenotypes, will be needed to define the spectrum of progenitor cell types in the adult brain. These considerations aside, multipotential and neurogenic progenitors are abundant in the adult human white matter and are both extractable and expandable. These cells may prove to be important agents for both induction and implantation strategies of cell-based neurological therapy.

### Methods

**Tissue dissociation and culture.** Adult subcortical white matter was surgically obtained from 21 patients, including 14 undergoing epileptic resections (age 1–50 years; 7 males and 7 females), one undergoing aneurysmal repair (69-year-old male), 2 undergoing resections of a noncontiguous dysplastic focus (20-year-old male and 36-year-old female) and 4 undergoing traumatic temporal lobe decompressions (17–67 years old; all males). Samples were obtained from patients who consented to tissue use under protocols approved by the New York Hospital–Cornell and Columbia Presbyterian Hospital Institutional Review Boards. The samples were dissected and dissociated to single-cell suspensions using papain and DNase as described<sup>23,34</sup>. The cells were then suspended in DMEM/F12/N1 with either bFGF (20 ng/ml; Sigma, St. Louis, Missouri) alone or bFGF with NT-3 (2 ng/ml; R&D Minneapolis, Minnesota) and PDGF-AA (20 ng/ml; Sigma), and plated in 100-mm suspension culture dishes (Corning, New York).

**Magnetic separation of A2B5<sup>+</sup> cells.** The number of viable cells was determined using calcein (Molecular Probes, Eugene, Oregon) 24–48 h after dissociation. The cells were then washed and incubated with A2B5 supernatant (clone 105; American Type Culture Collection, Manassas, Virginia) for 30–45 min at 4 °C, washed 3 times with PBS containing 0.5% BSA and 2 mM EDTA, and incubated with microbead-tagged mouse-specific rat IgM (1:4; Miltenyi Biotech, Bergisch Gladbach, Germany) for 30 min at 4 °C. The A2B5<sup>+</sup> cells were washed, resuspended and separated using positive selection columns, type MS<sup>+</sup> RS<sup>+</sup> or LS<sup>+</sup> VS<sup>+</sup> (magnetic cell sorting (MACS); Miltenyi Biotech). For flow cytometry of matched samples, cells were incubated in FITC-labeled mouse-specific goat IgM at 1:50 before FACS.

**Transfection and sorting.** Samples were transfected with pCNP2:hGFP after 2–6 d *in vitro*, using 2 µg of plasmid DNA and 10 µl of Lipofectin

(Gibco, Carlsbad, California) as described<sup>23,35</sup>. Sorting for pCNP2:hGFP and A2B5 immunofluorescence was performed on a Becton-Dickinson FACS Vantage (San Diego, California), also as described<sup>23,35</sup>. Untransfected and IgM-exposed control cells were used to calibrate background; a false-positive rate of 1% was accepted as cutoff.

**Generation of primary and secondary spheres.** A2B5<sup>+</sup> and A2B5-depleted white-matter cells were distributed to a 24-well plate directly after sorting, at 100,000, 50,000, 25,000, 10,000, 5,000 and 1,000 cells/ml with 0.5 ml/well of DMEM/F12/N1 with bFGF/NT3/PDGF-AA. The resulting WMPC-derived neurospheres were passaged at the 50- to 100-cell stage, by dissociation to single cells with trypsin and EDTA. The cells were plated at 3,000 cells/well. Three weeks later, the resultant secondary spheres were either dissociated and passaged again as tertiary spheres, or plated into 2% FBS with 20 ng/ml brain-derived neurotrophic factor on a polyornithine and fibronectin substrate and fixed 2 weeks later.

**Lentiviral tagging and lineage analysis.** A2B5-sorted cells were infected 2–5 d after separation with lentivirus (10<sup>8</sup> PFU/ml) expressing GFP under CMV promoter control and a WPRES post-transcriptional regulatory element<sup>12,13</sup>. The lentivirus was generated by co-transfecting plasmids pCMV/DR8.91, pMD.G, and pHRCMVGFp<sub>swin</sub> into 293T cells as described<sup>14</sup>. A2B5-sorted cells were exposed to lentivirus for 24 h in polybrene-supplemented medium (8 µg/ml), then passaged into fresh medium in 24-well plates. GFP expression by tagged cells was observed within 2 d. The primary spheres that arose in these cultures were dissociated 3 weeks later and replated at 3,000 cells/well; secondary spheres arose from these within 2 weeks.

**TRAP assay.** Telomerase activity was determined using the TRAP assay<sup>23,24</sup>, described in detail in the material accompanying Supplementary Figure 1 online.

**In utero transplantation.** Transuterine xenograft into E17 rat fetuses was performed as described<sup>21,36</sup>. Some cells were injected within 24–48 h after sorting and others after 10 d *in vitro* in FGF2, PDGF-AA and NT3. One month after implantation, the animals were perfusion-fixed by 4% paraformaldehyde. Experiments were conducted with the approval of the Institutional Animal Care and Use Committee of the Weill Medical College of Cornell University.

**Immunocytochemistry.** Xenografted rat brains were cryosectioned at 15 µm, permeabilized with PBS, 0.1% saponin and 1% NGS, and blocked with PBS, 0.05% saponin and 5% NGS, each for 30 min. Sections were labeled with HNA-specific mouse antibody (1:50; Chemicon, Temecula, California), then immunostained with βIII-tubulin-specific antibody TuJ1 (1:600; Covance, Princeton, New Jersey), MAP2-specific antibody AP-20 (1:50; Sigma), HuC/HuD-specific mouse monoclonal antibody 16A11 (25 µg/ml; H. Fumeaux, Memorial Sloan-Kettering Cancer Center, New York), GAD67-specific rabbit antibody (1:100; Chemicon), GFAP-specific mouse antibody SMI 21 (1:1,000; Sternberger, Lutherville, Maryland), GFAP-specific rabbit antibody (1:400; Sigma), CNP-specific mouse antibody SMI 91 (1:1000 Sternberger), human nestin-specific rabbit antibody (1:200; Chemicon), or doublecortin-specific rabbit antisera (1:100; C. Walsh, Harvard Medical School, Boston, Massachusetts). The sections were incubated with antibody overnight at 4 °C. Species- and isotype-specific fluorescent secondary antibodies were applied at 1:100 for 1.5 h at room temperature.

O4 and A2B5 were immunolabeled *in vitro* as described<sup>2</sup>. For multiple-antigen labeling, O4 was localized on live cells that were then fixed and stained for βIII-tubulin, MAP2, GFAP, Hu, GAD67 or BrdU. O4 supernatant (R. Bansal and S. Pfeiffer, University of Connecticut Health Center, Farmington, Connecticut) was used at 1:100 for 40 min at 4 °C. Antibodies against βIII-tubulin, MAP-2, GFAP and BrdU (BrdU-specific rat antibody; 1:200; Harlan, Indianapolis, Indiana) were incubated overnight at 4 °C. Fixed cultures were counterstained with DAPI (10 µg/ml; Molecular Probes).

**Confocal imaging.** In the xenografted brains, single cells that appeared co-labeled for both human- and cell-specific markers were evaluated by confocal imaging as described<sup>21,37</sup>. To be deemed double labeled, cells were

required to have HNA-specific signal surrounded by neuronal or glial immunoreactivity in every serially acquired 0.4- $\mu$ m z-dimension optical section, as well as in each orthogonal side view thereof.

**Calcium imaging.** Outgrowths from both first- and second-passage WMPC-derived neurospheres were assessed 2–3 weeks after plating into BDNF-supplemented DMEM/F12/N1 with 2% FBS. These mixed neuronal and glial outgrowths were challenged with 100  $\mu$ M glutamate or 60 mM potassium. Cytosolic calcium imaging was conducted using confocal microscopy of cultures loaded with Fluo-3 acetoxymethyl ester (Molecular Probes)<sup>33,38,39</sup>. We previously reported that adult progenitor-derived human neurons showed a mean calcium rise of >400% in response to 60 mM potassium *in vitro*, compared with glial responses of <20%<sup>38</sup>. In this study, we assigned neuronal identity to cells with  $\geq$ 2-fold calcium elevations to depolarization.

**Electrophysiology.** Sister cultures to those subjected to calcium imaging were assessed by whole-cell patch-clamp analysis. Whole-cell voltage-clamped recordings of fiber-bearing cells were conducted and analyzed as described<sup>15,33</sup>. A holding potential of –60 mV and voltage steps of 10 mV with 100-ms durations were applied to the recorded cells through the patch electrodes. Signals were sampled every 50  $\mu$ s.

*Note: Supplementary information is available on the Nature Medicine website.*

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#### Competing interests statement

The authors declare that they have no competing financial interests.

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- Scolding, N. *et al.* Oligodendrocyte progenitors are present in the normal adult human CNS and in the lesions of multiple sclerosis [see comments]. *Brain* **121**, 2221–2228 (1998).
- Roy, N.S. *et al.* Identification, isolation, and promoter-defined separation of mitotic oligodendrocyte progenitor cells from the adult human subcortical white matter. *J. Neurosci.* **19**, 9986–9995 (1999).
- Gravel, M., DiPolo, A., Valera, P. & Braun, P. Four-kilobase sequence of the mouse CNP gene directs spatial and temporal expression of lacZ in transgenic mice. *J. Neurosci. Res.* **53**, 393–404 (1998).
- Kondo, T. & Raff, M. Oligodendrocyte precursor cells reprogrammed to become multipotential CNS stem cells. *Science* **289**, 1754–1757 (2000).
- Palmer, T.D., Markakis, E.A., Willhoite, A.R., Safar, F. & Gage, F.H. Fibroblast growth factor-2 activates a latent neurogenic program in neural stem cells from diverse regions of the adult CNS. *J. Neurosci.* **19**, 8487–8497 (1999).
- Reynolds, B.A. & Weiss, S. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. *Science* **255**, 1707–1710 (1992).
- Vescovi, A.L., Reynolds, B.A., Fraser, D.D. & Weiss, S. bFGF regulates the proliferative fate of unipotent (neuronal) and bipotent (neuronal/astroglial) EGF-generated CNS progenitor cells. *Neuron* **11**, 951–966 (1993).
- Morshead, C.M. *et al.* Neural stem cells in the adult mammalian forebrain: a relatively quiescent subpopulation of subependymal cells. *Neuron* **13**, 1071–1082 (1994).
- Vescovi, A. *et al.* Isolation and cloning of multipotential stem cells from the embry-

- onic human CNS and establishment of transplantable human stem cells lines by epigenetic stimulation. *Exp. Neurol.* **156**, 71–83 (1999).
- Svendsen, C., Caldwell, M. & Ostenfeld, T. Human neural stem cells: isolation, expansion and transplantation. *Brain Pathol.* **9**, 499–513 (1999).
- Carpenter, M. *et al.* *In vitro* expansion of a multipotent population of human neural progenitor cells. *Exp. Neurol.* **158**, 265–278 (1999).
- Han, J. *et al.* Transgene expression in the guinea pig cochlea mediated by a lentivirus-derived gene transfer vector. *Hum. Gene Ther.* **10**, 1867–1873 (1999).
- Zufferey, R., Donello, J., Trono, D. & Hope, H. Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. *J. Virol.* **73**, 2886–2892 (1999).
- Windrem, M. *et al.* Progenitor cells derived from the adult human subcortical white matter disperse and differentiate as oligodendrocytes within demyelinated regions of the rat brain. *J. Neurosci. Res.* **69**, 966–975 (2002).
- Kang, J., Jiang, L., Goldman, S.A. & Nedergaard, M. Astrocyte-mediated potentiation of inhibitory synaptic transmission. *Nat. Neurosci.* **1**, 683–692 (1998).
- Tse, F.W., Fraser, D.D., Duffy, S. & MacVicar, B.A. Voltage-activated K<sup>+</sup> currents in acutely isolated hippocampal astrocytes. *J. Neurosci.* **12**, 1781–1788 (1992).
- Barres, B.A. *et al.* A crucial role for neurotrophin-3 in oligodendrocyte development. *Nature* **367**, 371–375 (1994).
- Raff, M.C., Lillien, L.E., Richardson, W.D., Bume, J.F. & Noble, M.D. Platelet-derived growth factor from astrocytes drives the clock that times oligodendrocyte development in culture. *Nature* **333**, 562–565 (1988).
- Vicario-Abejon, C., Johe, K.K., Hazel, T.G., Collazo, D. & McKay, R.D. Functions of basic fibroblast growth factor and neurotrophins in the differentiation of hippocampal neurons. *Neuron* **15**, 105–114 (1995).
- Johe, K.K., Hazel, T.G., Muller, T., Dugich-Djordjevic, M.M. & McKay, R.D. Single factors direct the differentiation of stem cells from the fetal and adult central nervous system. *Genes Dev.* **10**, 3129–3140 (1996).
- Keyoung, H.M. *et al.* Specific identification, selection and extraction of neural stem cells from the fetal human brain. *Nat. Biotechnol.* **19**, 843–850 (2001).
- Yashima, K. *et al.* Expression of the RNA component of telomerase during human development and differentiation. *Cell Growth Differ.* **9**, 805–813 (1998).
- Ostenfeld, T. *et al.* Human neural precursor cells express low levels of telomerase *in vitro* and show diminishing cell proliferation with extensive axonal outgrowth following transplantation. *Exp. Neurol.* **164**, 215–226 (2000).
- Kim, N. *et al.* Specific association of human telomerase activity with immortal cells and cancer. *Science* **266**, 2011–2015 (1994).
- Gleeson, J., Lin, P., Flanagan, L. & Walsh, C. Doublecortin is a microtubule-associated protein and is expressed widely by migrating neurons. *Neuron* **23**, 257–271 (1999).
- Doetsch, F., Caille, I., Lim, D., Garcia-Verdugo, J. & Alvarez-Buylla, A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell* **97**, 703–716 (1999).
- Alvarez-Buylla, A. & Garcia-Verdugo, J.M. Neurogenesis in adult subventricular zone. *J. Neurosci.* **22**, 629–634 (2002).
- Kukekov, V., Laywell, E., Thomas, L. & Steindler, D. A nestin-negative precursor cell from the adult mouse brain gives rise to neurons and glia. *Glia* **21**, 399–407 (1997).
- Levine, J.M., Reynolds, R. & Fawcett, J.W. The oligodendrocyte precursor cell in health and disease. *Trends Neurosci.* **24**, 39–47 (2001).
- Mason, J. & Goldman, J. A2B5<sup>+</sup> and O4<sup>+</sup> cycling progenitors in the adult forebrain white matter respond differentially to PDGF-AA, FGF-2, and IGF-1. *Molec. Cell. Neurosci.* **20**, 30–42 (2002).
- Jiang, Y. *et al.* Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature* **418**, 41–49 (2002).
- Capela, A. & Temple, S. LeX/sox-1 is expressed by adult mouse CNS stem cells, identifying them as nonependymal. *Neuron* **35**, 865–875 (2002).
- Roy, N.S. *et al.* *In vitro* neurogenesis by progenitor cells isolated from the adult human hippocampus. *Nat. Med.* **6**, 271–277 (2000).
- Roy, N.S. *et al.* Promoter-targeted selection and isolation of neural progenitor cells from the adult human ventricular zone. *J. Neurosci. Res.* **59**, 321–331 (2000).
- Wang, S. *et al.* Isolation of neuronal precursors by sorting embryonic forebrain transfected with GFP regulated by the T $\alpha$ 1 tubulin promoter. *Nat. Biotechnol.* **16**, 196–201 (1998).
- Brustle, O. *et al.* Chimeric brains generated by intraventricular transplantation of fetal human brain cells into embryonic rats. *Nat. Biotechnol.* **16**, 1040–1044 (1998).
- Benraiss, A., Chmielnicki, E., Roh, D. & Goldman, S.A. Adenoviral BDNF induces both neostriatal and olfactory neuronal recruitment from endogenous progenitor cells in the adult forebrain. *J. Neurosci.* **21**, 6718–6731 (2001).
- Kirschenbaum, B. *et al.* *In vitro* neuronal production and differentiation by precursor cells derived from the adult human forebrain. *Cereb. Cortex* **4**, 576–589 (1994).
- Pincus, D.W. *et al.* FGF2/BDNF-associated maturation of new neurons generated from adult human subependymal cells. *Ann. Neurol.* **43**, 576–585 (1998).
- Marusich, M., Furneaux, H., Henion, P. & Weston, J. Hu neuronal proteins are expressed in proliferating neurogenic cells. *J. Neurobiol.* **25**, 143–155 (1994).
- Barami, K., Iversen, K., Furneaux, H. & Goldman, S.A. Hu protein as an early marker of neuronal phenotypic differentiation by subependymal zone cells of the adult songbird forebrain. *J. Neurobiol.* **28**, 82–101 (1995).





## Plasmodium falciparum during pregnancy: a puzzling parasite

Continuing my interest on cell biology I pursued a post-doc focusing on *Plasmodium Falciparum*. During my post-doctoral training at the Institut Pasteur I developed projects dedicated to identify molecules expressed by *Plasmodium Falciparum* at the surface of the human infected red blood cells that could be used as vaccine candidates for prevention of pregnancy malaria. A focus of my research was the mechanisms of antigenic variation and switching of expression of variant surface molecules in *P. Falciparum* through which the parasite is able to evade immune pressure and infect the human placenta (M. C. Nunes and Scherf 2007; Marta C. Nunes et al. 2008). I characterized a novel protein-kinase family in *P. falciparum*, the FIKK-proteins. Since the genes encoding these proteins locate mostly in the chromosomal sub-telomeric regions; close to other genes that play a role in remodelling the infected erythrocyte membrane, I hypothesized that the FIKK-proteins might be involved in specific host-parasite interactions. By creating knock-out parasites and comparing the relative mRNA levels of the FIKK-genes between parasites expressing different surface proteins I detected quantitative and qualitative biological differences for several surface genes (Marta C. Nunes et al. 2007). Since the FIKK-kinase family is Apicomplexa-specific the knowledge acquired from this project could be used to develop drugs or vaccines targeting these kinases (Marta C. Nunes et al. 2010).

During my post-doc I co-authored six papers (Lopez-Rubio et al. 2007; Marta C. Nunes et al. 2007; 2008; 2010; M. C. Nunes and Scherf 2007; Viebig et al. 2006); in four of them I was first author and in one I was the corresponding author. I presented my work at four international conferences. To develop my work, I was awarded with two competitive international fellowships: European Molecular Biology Organization (EMBO), Post-doctoral long-term Fellowship; and Human Frontiers Science Program, Post-doctoral Fellowship.

During my post-doctoral training I became interested in epidemiology and infectious diseases.

Below the paper describing the protein kinase family of *Plasmodium Falciparum* that targets distinct components of the host erythrocyte membrane.

## A novel protein kinase family in *Plasmodium falciparum* is differentially transcribed and secreted to various cellular compartments of the host cell

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### Summary

Processes at the surface of *Plasmodium falciparum*-infected erythrocytes such as antigenic variation and cytoadhesion may be modulated by active signalling between host and parasite. Potential candidates for this role include the putative kinases of the FIKK family. The novel *Apicomplexa*-specific FIKK gene has expanded in *P. falciparum* to 20 sequence-related members distributed between 11 chromosomes. Specific antibodies raised against different members indicated that most FIKK proteins locate to punctate foci in the erythrocyte cytoplasm that colocalized with Maurer's clefts proteins. One FIKK member dissociates at the trophozoite stage from the Maurer's clefts and relocates with the erythrocyte cytoskeleton. Another FIKK protein, despite having a PEXEL motif, remains located within the parasite. FIKK proteins possess the essential residues for phosphotransferase activity. We show that protein kinase activity was detected in immunoprecipitates obtained with two anti-FIKK antibodies. Quantitative PCR analysis revealed differential gene transcription of the FIKK paralogues in asexual blood stages parasites. We observed significant changes in the transcription pattern between parasites with different adhesion phenotypes. Our data suggest a role of FIKK proteins in the remodelling of the erythrocyte surface and reveal the existence of an adaptable parasite system able to sense intra- and possibly extracellular changes.

### Introduction

Malaria is one of the most important parasitic infections, causing major challenges to health and socio-economic development in poor countries. *Plasmodium falciparum* is the parasite responsible for the most severe form of the disease in humans. The particular virulence of this organism is due in part to the way in which it modifies the membrane of the infected erythrocytes (IEs) in which it grows and multiplies, through export of remodelling factors and several variable antigens. These virulence factors involved in host–parasite interactions are encoded by members of multigene families mostly located in the chromosomal subtelomeric regions (for review see Kyes *et al.*, 2001). The best characterized is the polymorphic *var* multigene family, which encodes a large, highly variant parasite cytoadhesion molecule called *Plasmodium falciparum* erythrocyte membrane protein 1 (PfEMP1) (Baruch *et al.*, 1995; Smith *et al.*, 1995; Su *et al.*, 1995). PfEMP1 molecules are trafficked to the outside of the parasite and inserted into the IE plasma membrane, where they mediate adhesion to a variety of host receptors on the vascular endothelium and other cells. There are approximately 60 *var* genes per haploid genome, which are predominantly situated in the subtelomeric regions of all 14 chromosomes (Gardner *et al.*, 2002). A single variant of the *var* gene family is expressed at the surface of an IE at a given time, and determines the adhesion to a host receptor. The switching in expression between the different *var* genes allows the parasite to undergo clonal antigenic variation, thus evading the host's immune response.

During first pregnancy a particular *var* gene called *var2CSA* is activated (Salanti *et al.*, 2003; Gamain *et al.*, 2005; Viebig *et al.*, 2005), leading to the massive sequestration of IEs in the placenta through the binding to chondroitin sulphate A (CSA) expressed by syncytiotrophoblastic cells (Fried and Duffy, 1996). Thus, the placenta constitutes a site which favours the expression of antigenically distinct parasites. It is tempting to speculate that host factors can modulate the expression of variant antigens at the surface of IEs. Although the underlying mechanisms remain unclear, it is suggested to require active signalling between host and parasite (Brown, 1973; Barnwell *et al.*, 1983; David *et al.*, 1983).

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An *in silico* search for candidate genes that could be involved in sensing internal or external changes in the IE led us to consider an intriguing, newly discovered *Apicomplexa*-specific protein kinase family. Genome data base mining has recently revealed a novel putative kinase family in *P. falciparum* called the FIKK family, on the basis of the conserved phenylalanine, isoleucine, lysine, lysine amino acid motif (Ward *et al.*, 2004; Kooij *et al.*, 2005; Schneider and Mercereau-Puijalon, 2005). A single copy gene in most *Plasmodium* species has expanded in *P. falciparum* to 20 related members distributed throughout 11 chromosomes. In *P. falciparum* most of the FIKK paralogues are located in the chromosomal subtelomeric regions, close to other genes that are differentially expressed and play a role in remodelling the IE membrane. Synteny maps of *P. falciparum* and other *Plasmodium* species show that most of the FIKK genes, like other species-specific genes, are located in the regions of breakpoint in synteny (Kooij *et al.*, 2005). The resulting subtelomeric location in *P. falciparum* most likely has enhanced gene diversification and amplification through mechanisms of ectopic recombination that are facilitated by the clustering of telomeres at the nuclear periphery (Freitas-Junior *et al.*, 2000). FIKK proteins might therefore be involved in specific host–parasite interactions such as the regulation of expression of the *var* gene family.

In addition to a conserved eukaryotic kinase domain-like region, the FIKK sequences all have a highly variable N-terminal extension. Almost all 20 *P. falciparum* paralogues possess the *Plasmodium* exported element (PEXEL) and/or the vacuolar transport signal downstream of a signal or anchor sequence required for transport across the parasitophorous vacuole (Hiller *et al.*, 2004; Marti *et al.*, 2004). Due to the restricted homology with well-characterized kinase domains the FIKK proteins did not cluster within any of the kinase groups described in higher eukaryotes (Hanks, 2003).

In this work we investigated the expression and location of members of the FIKK putative kinase family. We show that they localize to distinct cellular compartments within the IE, including the erythrocyte membrane, Maurer's clefts and intracellular parasite. We also present experimental evidence that at least some of the FIKK proteins are associated with kinase activity and that the transcription levels of particular FIKK genes changes due to intracellular alterations associated with adhesion to CSA.

## Results

### Organization of the *P. falciparum* FIKK kinase gene family

We were interested to investigate potential signal transduction events in the IE that would modulate PfEMP1

expression during pregnancy in *P. falciparum*. Searching for candidate genes that could be relevant for this modulation, we detected a putative kinase gene (*PFL0040c*) next to the *var* gene expressed during pregnancy (*var2CSA*, *PFL0030c*). A search for homologues of the *PFL0040c* protein sequence in the *Plasmodium* genome research database (<http://www.plasmodb.org/plasmo/home.jsp>) found 19 additional annotated proteins that appeared to form a tight cluster in *P. falciparum*, with *E*-values from 3.4e-108 to 3.7e-39 (see Table 1). In our initial search an additional gene was found (*MAL13P1.109*); however, this gene no longer exists in the database. *MAL8P1.203* was not found in our initial search though it appears in PlasmoDB 4.4 version. This multigene group was identified independently in a genome-wide characterization of protein kinase-encoding genes and initially called the FIKK putative kinase family, based on an amino acid motif present in subdomain I of members of this family (Ward *et al.*, 2004). We will use this nomenclature and we propose to name each gene of the family accordingly to its chromosomal location (see Table 1).

Seventeen FIKK genes are located in the highly variable subtelomeric regions of 11 different chromosomes. Nineteen paralogues have a three-exon structure with a signal or anchor sequence (except for *FIKK9.2*, that has no signal sequence) followed by the PEXEL (Hiller *et al.*, 2004; Marti *et al.*, 2004; Schneider and Mercereau-Puijalon, 2005). In contrast the single orthologues from most other *Plasmodium* species lacks the first exon encoding the signal sequence. *FIKK8* lacks the first exon and the PEXEL motif, phylogenetic analyses group this gene in the same clade as the orthologues from the rodent species with single FIKK members (Kooij *et al.*, 2005). Most likely the FIKK family arose from recombination of this progenitor gene with an early relocation to a subtelomeric region, extensive duplication and PEXEL acquisition. FIKK4.2 has been identified previously as a *P. falciparum* antigen and named R45 trophozoite antigen (Bonney *et al.*, 1992; Schneider and Mercereau-Puijalon, 2005).

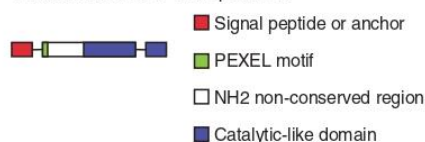
### FIKK proteins are exported to the red blood cell

We generated a C-terminal fusion of the FIKK12 (*PFL0040c*) protein with the reporter molecule green fluorescent protein (GFP) using the plasmid vector pARL (Crabb *et al.*, 2004). DNA sequence analysis of the cDNA of FIKK12 confirmed the existence of a three-exon structure; however, the first exon is not that predicted in PlasmoDB for the *PFL0040c* gene model. The full-length FIKK12 gene was joined upstream of the GFP coding sequence and the resultant GFP fusion protein was expressed under the control of the chloroquine-resistance transporter promoter (PfCRT) from stably maintained episomes. The FIKK12-GFP chimera was first detected at

**Table 1.** Summary of FIKK protein characteristics and of the antibodies used in our study.

| Annotated gene name | FIKK name          | Chromosome | Approximate distance from telomere (kb) | Observations                   | Antibodies       | Location in the IE                   |
|---------------------|--------------------|------------|---|--------------------------------|------------------|--------------------------------------|
| <i>PFA0130c</i>     | <i>FIKK1</i>       | 1          | 120                                     |                                |                  |                                      |
| <i>PFC0060c</i>     | <i>FIKK3</i>       | 3          | 75                                      |                                |                  |                                      |
| <i>PFD1165w</i>     | <i>FIKK4.1</i>     | 4          | 90                                      |                                | Guinea pig serum | Maurer's cleft                       |
| <i>PFD1175w</i>     | <i>FIKK4.2/R45</i> | 4          | 82                                      |                                |                  |                                      |
| <i>PFE0045c</i>     | <i>FIKK5</i>       | 5          | 52                                      |                                |                  |                                      |
| <i>MAL7P1.144</i>   | <i>FIKK7.1</i>     | 7          | 346                                     | 5' exon was predicted manually |                  |                                      |
| <i>MAL7P1.175</i>   | <i>FIKK7.2</i>     | 7          | 91                                      | Internal stop codon            |                  |                                      |
| <i>MAL8P1.203</i>   | <i>FIKK8</i>       | 8          | 320                                     | No exon 1                      |                  |                                      |
| <i>PFI0095c</i>     | <i>FIKK9.1</i>     | 9          | 91                                      |                                |                  |                                      |
| <i>PFI0100c</i>     | <i>FIKK9.2</i>     | 9          | 95                                      | No signal peptide              | Chicken antibody | Parasite                             |
| <i>PFI0105c</i>     | <i>FIKK9.3</i>     | 9          | 100                                     |                                | Guinea pig serum | Maurer's cleft                       |
| <i>PFI0110c</i>     | <i>FIKK9.4</i>     | 9          | 104                                     |                                |                  |                                      |
| <i>PFI0115c</i>     | <i>FIKK9.5</i>     | 9          | 108                                     |                                |                  |                                      |
| <i>PFI0120c</i>     | <i>FIKK9.6</i>     | 9          | 111                                     |                                | Rat serum        | Maurer's cleft                       |
| <i>PFI0125c</i>     | <i>FIKK9.7</i>     | 9          | 115                                     |                                |                  |                                      |
| <i>PF10_0160</i>    | <i>FIKK10.1</i>    | 10         | 659                                     |                                |                  |                                      |
| <i>PF10_0380</i>    | <i>FIKK10.2</i>    | 10         | 115                                     |                                |                  |                                      |
| <i>PF11_0510</i>    | <i>FIKK11</i>      | 11         | 51                                      | 2 PEXEL                        |                  |                                      |
| <i>PFL0040c</i>     | <i>FIKK12</i>      | 12         | 71                                      |                                | Chicken antibody | Maurer's cleft/ erythrocyte membrane |
| <i>PF14_0733/4</i>  | <i>FIKK14</i>      | 14         | 150                                     | Internal stop codon            | GFP tagging      |                                      |

General structure of FIKK proteins.



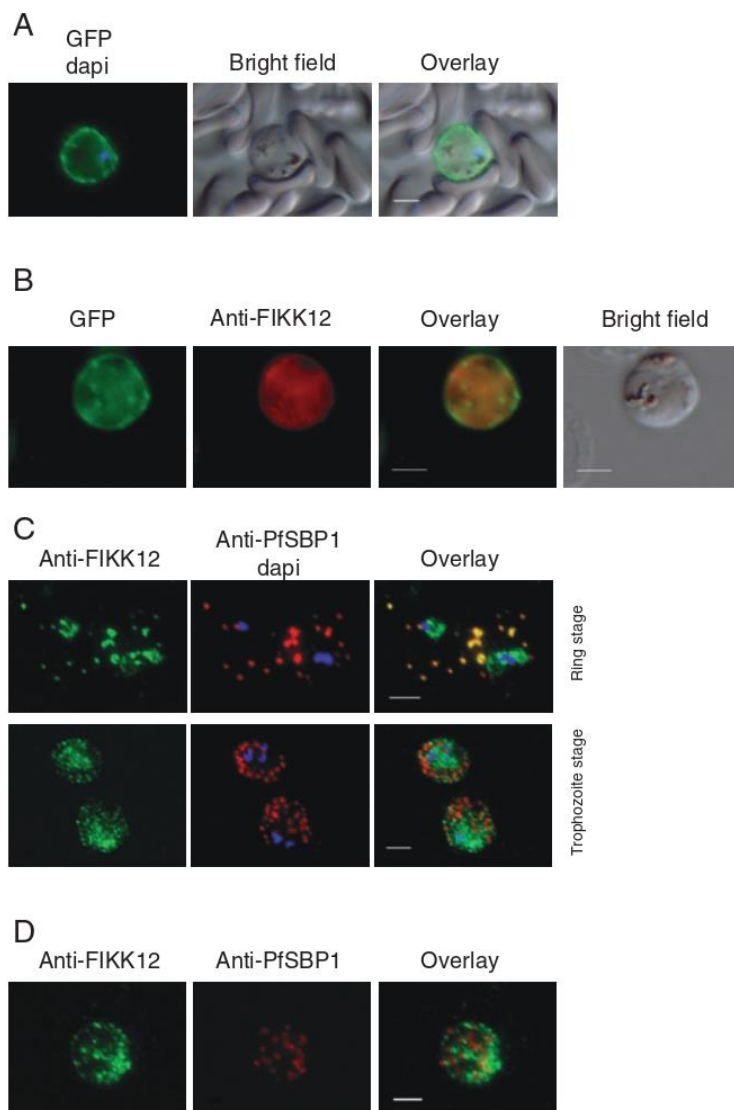
approximately 24 h after invasion, i.e. at the early trophozoite stage. FIKK12-GFP was transported outside of the parasitophorous vacuole to the host cell as expected from the presence of a PEXEL motif (see above). Fluorescence was detected as a diffuse pattern in the erythrocyte cytosol, and a rim-like signal at the periphery of the infected cells suggests that it is associated with the IE membrane (Fig. 1A and Fig. S1).

As GFP reporter proteins can be liable to location artefacts, we raised antibodies against a peptide from the FIKK12 protein to verify the predicted localization pattern of the GFP fusion protein. We performed a suspension immunofluorescence assay (IFA) protocol developed by Tonkin *et al.* (2004) that very well preserves the cellular integrity and maintains GFP fluorescence. By IFA this antibody recognized punctate spots outside of the parasitophorous vacuole throughout the cytoplasm of the IE. The antibody signal colocalized with the FIKK12-GFP protein at the periphery of the IE (Fig. 1B). However, not all the vesicular structures recognized by the specific antibody were GFP-tagged. It appeared that the chimeric protein has a preference to associate with the periphery of the IE.

Many parasite proteins exported to the red blood cell have been localized to intraerythrocytic structures called the Maurer's clefts (Stanley *et al.*, 1989; Hinterberg

*et al.*, 1994), which appear in IFA as punctate dots. To determine whether FIKK12 associated with Maurer's clefts we used antibodies against the antigens Pf332 (Hinterberg *et al.*, 1994) (data not shown) and *P. falciparum* skeleton binding protein 1 (PfSBP1) (Blisnick *et al.*, 2000), two proteins previously described as being associated with Maurer's clefts. In ring stage-infected erythrocytes, FIKK12 colocalized with vesicle-like structures stained with anti-PfSBP1 (Fig. 1C). However, at the trophozoite stage FIKK12 dissociated from the Maurer's clefts and apparently relocated to a distinct location compatible with the erythrocyte cytoskeleton (Fig. 1C). To confirm this observation, we prepared ghosts from trophozoite stage-infected erythrocytes and performed an IFA using the FIKK12 antibody. PfSBP1 was used as a positive control to confirm the presence of Maurer's clefts in ghosts (Blisnick *et al.*, 2000). In the ghost preparation we obtained a spotted pattern signal (like in intact cells) that did not colocalize with our Maurer's cleft marker, showing that FIKK12 is indeed located at the erythrocyte membrane (Fig. 1D).

To further characterize the cellular location of FIKK proteins we raised peptide-derived antibodies against four other members of the FIKK family (FIKK4.1, FIKK9.2, FIKK9.3, FIKK9.6). We used three different



**Fig. 1.** Analysis of expression and localization of FIKK12 protein.

A. Live 3D7 *P. falciparum* trophozoite stage-infected erythrocytes expressing chimeric FIKK12-GFP protein. Strong GFP fluorescence is typically seen in trophozoite and schizont stages. Fusion protein is trafficked to the erythrocyte cytoplasm and concentrates at the erythrocyte plasma membrane.

B. Immuno-localization of the GFP chimera in transgenic parasites. First image GFP fluorescence, second image cells incubated with anti-FIKK12 antibody, third image overlay of first and second image showing that FIKK12-GFP colocalizes with anti-FIKK12 at the surface of the IEs.

C and D. Transient colocalization of FIKK12 to Maurer's cleft assessed by air dried immunofluorescence using anti-FIKK12 (green) and anti-PfSBP1 (red) antibodies in 3D7 parasites.

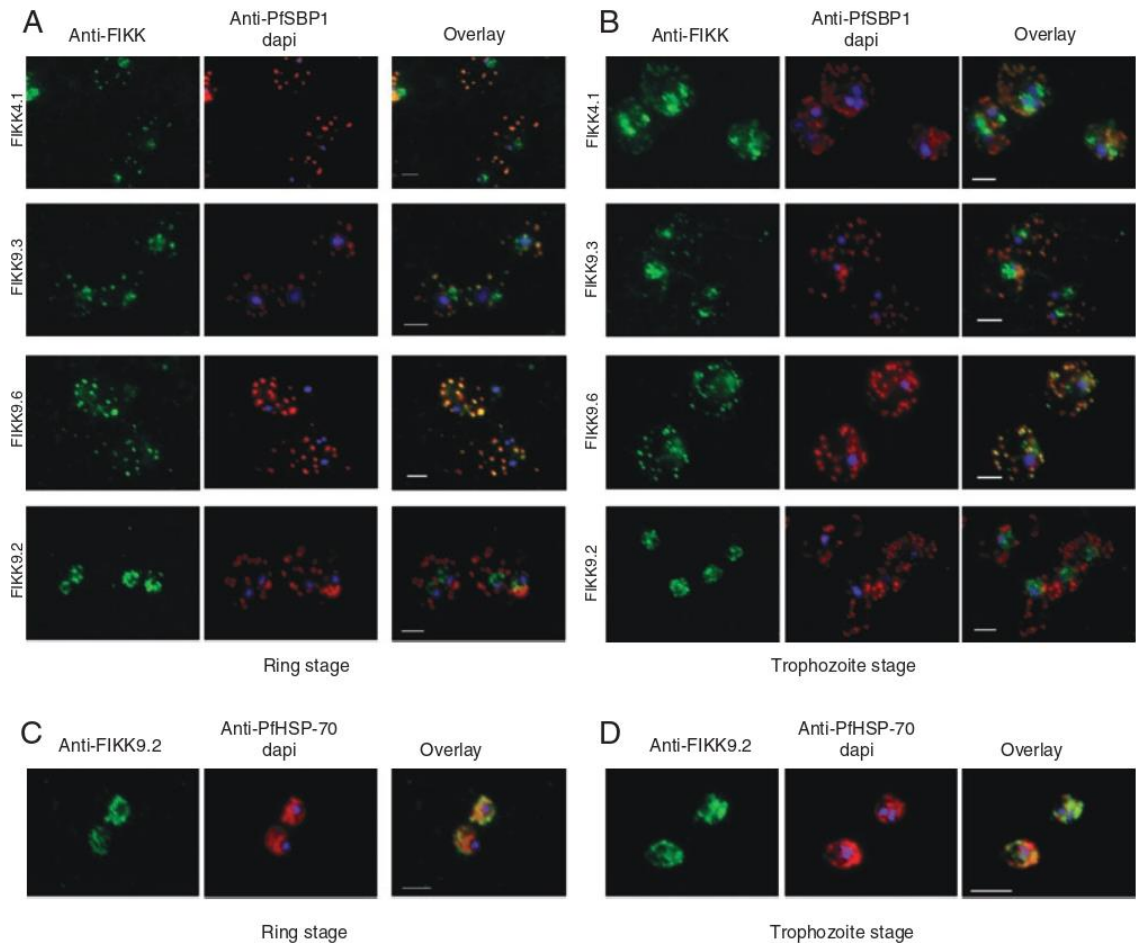
C. First row: colocalization of FIKK12 with PfSBP1 in ring stage *P. falciparum*-infected erythrocytes. Second row: colocalization is lost at the trophozoite stage.

D. Trophozoite stage *P. falciparum*-infected erythrocyte ghosts. Scale bars, 3  $\mu$ m. Nuclei of *P. falciparum* detected by DAPI staining (blue) in all the figures.

sources of antibody (guinea pig and rat sera, chicken IgY affinity-purified). Immunofluorescence revealed punctate structures outside of the parasite throughout the cytoplasm of the IE, resembling Maurer's clefts for FIKK4.1, FIKK9.3 and FIKK9.6, but not for FIKK9.2 (Fig. 2A and B). To determine whether the foci within the IE cytosol were associated with Maurer's clefts we tested colocalization with PfSBP1. Immunofluorescence confirmed that FIKK4.1, FIKK9.3 and FIKK9.6 colocalized with PfSBP1 in the Maurer's clefts in early ring, trophozoite and schizont stage parasites (data not shown). These proteins are thus associated with Maur-

er's clefts structures throughout the parasite intraerythrocytic cycle.

FIKK9.2 antibody recognized a protein that was not transported beyond the parasite membrane. To confirm this subcellular localization we used PfHSP-70 antibody, a known intraparasitic marker (Mattei *et al.*, 1989). FIKK9.2 colocalized with PfHSP-70 in ring and trophozoite stage-infected erythrocytes (Fig. 2C and D). FIKK9.2 has no signal peptide even though it has the PEXEL motif. The NH<sub>2</sub>-terminal sequence of this protein is apparently not sufficient to cause the protein to enter the secretory system.



**Fig. 2.** Localization by immunofluorescence of FIKK4.1, FIKK9.2, FIKK9.3 and FIKK9.6 proteins in 3D7 parasites. A and B. Ring stage (A) and trophozoite stage (B) *P. falciparum*-infected erythrocytes were air dried and immunolabelled with PfSBP1 antibody (red) and a FIKK-specific antibody (green). First row: polyclonal guinea pig anti-FIKK4.1; second row: polyclonal guinea pig anti-FIKK9.3; third row: polyclonal rat anti-FIKK9.6; last row: chicken anti-FIKK9.2. The third column in each panel represents the merging of the red and green channels. Yellow areas represent regions of colocalization seen on both parasitic stages for all the FIKK proteins except FIKK9.2. C and D. FIKK9.2 (green) colocalization inside the parasite with PfHSP-70 (red) in ring stage (C) and trophozoite stage (D) *P. falciparum*-infected erythrocytes. Scale bars, 3  $\mu$ m. Nuclei of *P. falciparum* detected by DAPI staining (blue) in all the figures.

To confirm the specificity of the signal obtained with the unpurified sera IFA was undertaken in the presence of competing epitopes. The FIKK signal intensity decreased with increasing concentrations of the specific peptides. In contrast, when non-specific peptides were used as competitor, the FIKK signal intensity remained unchanged (see Fig. S2).

#### *FIKK proteins have protein kinase activity*

Reversible phosphorylation catalysed by protein kinases and phosphatases of specific proteins is a very important mechanism for the regulation of protein and enzyme activ-

ity in the transduction of developmental, metabolic and environmental cues. Protein kinases, though diverse, all share a common catalytic domain which typically extends for about 260 amino acid residues, including the regions for ATP binding, protein substrate recognition and catalysis (Hanks *et al.*, 1988; Knighton *et al.*, 1991). The FIKK proteins contain a kinase catalytic domain with the highly conserved regions involved in phosphotransfer, amino acid targeting and catalysis, although they lack a glycine-rich loop for ATP anchoring in subdomain I (Ward *et al.*, 2004). Nevertheless it has been established that ATP binding and phosphotransfer ability is not absolutely dependent on the presence of the glycine triad (Dorin

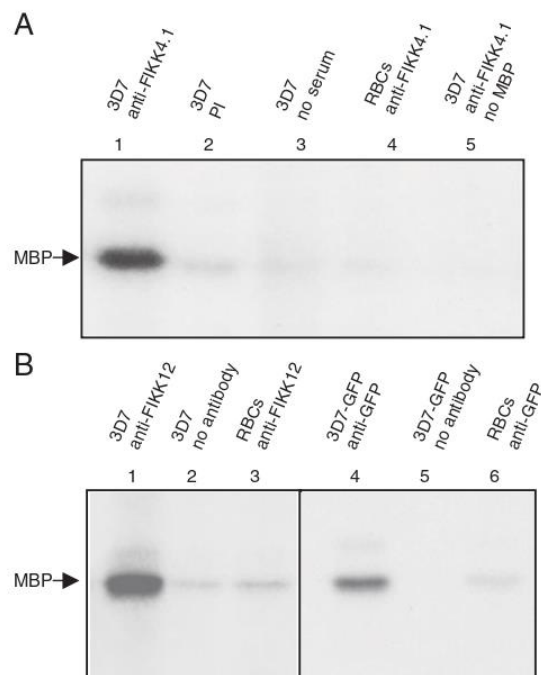
*et al.*, 2005). We therefore investigated if these proteins could in fact behave as functional kinases.

We used peptide-derived antibodies against FIKK4.1 and FIKK12 to immunoprecipitate the putative kinases from IE lysates. An anti-GFP antibody was used as well to immunoprecipitate the chimeric FIKK12-GFP protein from transgenic parasite lysates. The immunoprecipitated complexes were then tested in an *in vitro* kinase reaction. We tested for phosphorylation of the exogenous substrate myelin basic protein (MBP) and with FIKK4.1 for auto-phosphorylation (i.e. no exogenous substrate was added to the kinase reaction). Pre-immune serum, no primary antibody and extracts from non-infected erythrocytes served as control. Comparing the basal level of substrate phosphorylation from non-infected erythrocytes, omitting the primary antibody or using the pre-immune serum with the signal exhibited by the FIKK immunocomplex, we could detect a large increase in substrate phosphorylation by the latter (Fig. 3A and B). Both native FIKK12 and FIKK12-GFP immunocomplexes were able to phosphorylate MBP certifying that the GFP tagging did not disturb the activity of this protein (Fig. 3B).

The precipitated kinases used MBP as a substrate, but autophosphorylation was not detected under the conditions used in our assay. These kinases had been classified as non-RD kinases, they therefore are not expected to require phosphorylation of the activation segment for activation (Anamika *et al.*, 2005). Outside of the catalytic domain FIKK kinases have no homology with regulatory domains of other protein kinases, indicating that activity of these kinases might be modulated in a different way from those of higher eukaryotes.

#### Transcription levels of *P. falciparum* FIKK genes during parasite blood stages and association with *var* gene expression

We next investigated if all the members of the *FIKK* gene family were transcribed in asexual blood stage parasites. To monitor *FIKK* genes transcription levels we used quantitative real-time reverse transcriptase polymerase chain reaction analysis using specific pairs of primers for all the *FIKK* genes (see list of primers in Table S1). Parasite RNA was harvested at three different time points of the 48 h (ring stage 10–14 h, trophozoite stage 26–30 h, schizont stage 40–46 h) intraerythrocytic cycle from FCR3 strain *P. falciparum* selected to adhere *in vitro* to the CD36 receptor (FCR3CD36). We compared the transcription levels of each *FIKK* gene to the transcription levels of two housekeeping genes, the *calmodulin* gene (*PF14\_0323*) and the *seryl-tRNA synthetase* gene (*PF07\_0073*) (data not shown). We observed that during the intraerythrocytic cycle the relative transcription levels of the different *FIKK* genes were very different. Transcription was highest at



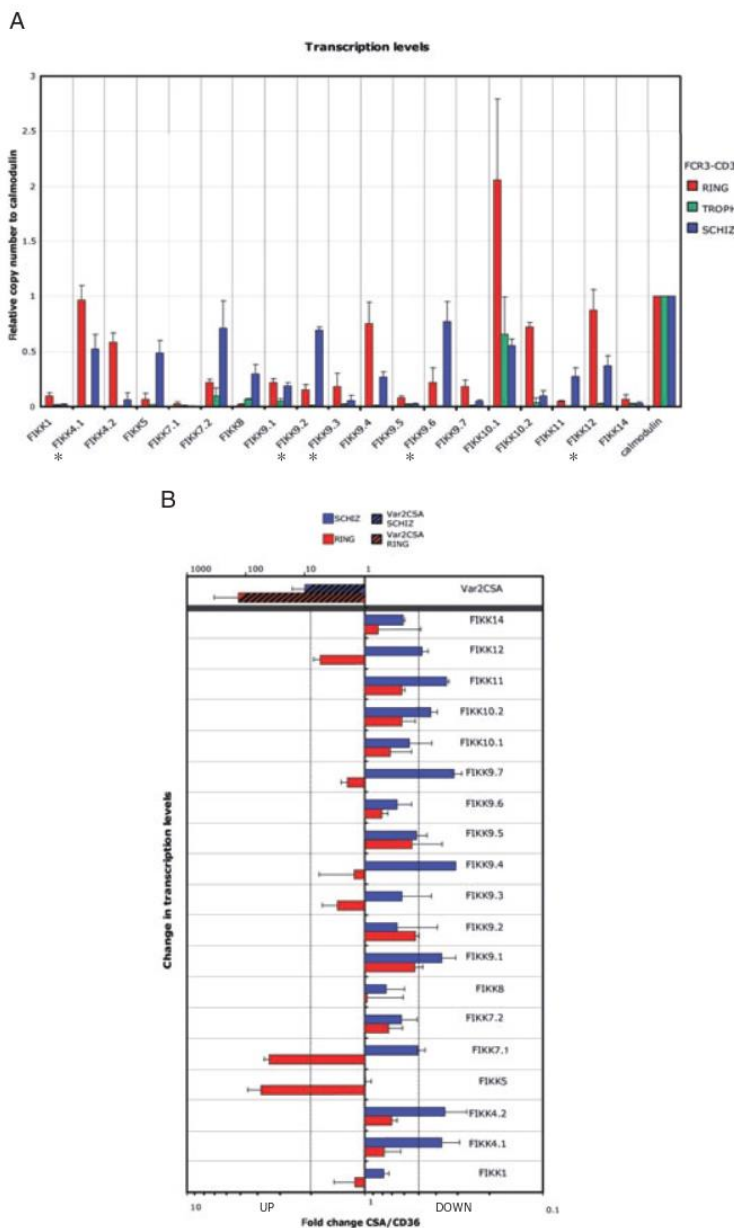
**Fig. 3.** FIKK4.1, FIKK12 and FIKK12-GFP kinase activity after immunoprecipitation. *In vitro* kinase reactions were carried out to test kinase activity of FIKK immunoprecipitated proteins. Assays were performed in the presence of [ $\gamma$ - $^{32}$ P]-ATP and MBP as an artificial substrate.

A. Phosphorylation of MBP by FIKK4.1 immunocomplexes. Pre-immune serum (PI) (lane 2), no addition of serum (lane 3) and non-infected red blood cells (RBCs) (lane 4) were used in the immunoprecipitation as controls. In lane 5 MBP was omitted to test autophosphorylation.

B. Phosphorylation of MBP by FIKK12 immunocomplexes using antibody against native protein (left panel) or against GFP-tagged FIKK12 (right panel). No addition of antibody (lanes 2 and 5) and non-infected red blood cells (lanes 3 and 6) were used as controls. Samples were separated in a 12% SDS-polyacrylamide gel and submitted to autoradiography.

ring and schizont stages compared with trophozoite stage. Compared with our internal control calmodulin, whose transcription levels were arbitrarily set to unit one, approximately half of the genes were transcribed at similar levels of this housekeeping gene ( $\pm$  factor 2). The others were transcribed at lower levels (see Fig. 4A). A similar pattern was observed using RNA from 3D7 strain parasites (data not shown), suggesting that the overall profile is in some way hardwired.

Our initial hypothesis was that the FIKK proteins might be involved in regulation of *var* gene expression. To test this idea we compared the relative transcriptional levels of the *FIKK* genes between parasites expressing different *var* genes. FCR3 strain parasites were selected to adhere *in vitro* to CSA or to CD36 receptors as described previ-



**Fig. 4.** Real-time PCR analysis of transcription levels of the entire FIKK family. All values are represented as relative copy number to the housekeeping gene *calmodulin*. FCR3CSA- and FCR3CD36-selected parasites RNA was extracted from three different time points of the intraerythrocytic cycle.

A. FIKK family transcription profile using FCR3CD36 parasites. For the genes marked with asterisk (\*) expression was validated by IFA using specific antibodies. All data represent mean  $\pm$  SD.

B. Change in transcription (log scale) of the FIKK genes in ring and schizont stage-infected erythrocytes, following CSA selection. A twofold change in transcript abundance (dashed lines) was arbitrarily defined as the cut-off. *var2CSA* (*PFL0030c*) is shown as a highly transcribed gene in FCR3CSA ring stage-infected erythrocytes. Numbers on the top axis concern only the *var2CSA* gene. Numbers on the bottom axis concern all the FIKK genes.

ously (Scherf *et al.*, 1998). Comparing the relative mRNA levels of the FIKK genes between FCR3CSA- and FCR3CD36-selected parasites we detected differences higher than twofold for many genes (Fig. 4B and Fig. S3). Surprisingly, most observed changes in FCR3CSA lead to the downregulation of FIKK genes (eight genes). In contrast FIKK5 and FIKK7.1 were more than threefold upregulated in FCR3CSA-selected parasites. The magnitude of the differences observed at the transcription level

for the FIKK genes is much smaller than the transcriptional differences observed for *var* genes.

### Discussion

In this work we demonstrate that members of a novel *P. falciparum* multigene kinase family are exported to different locations in the IE. Phosphorylation is an efficient way of reversibly changing the biochemical properties of



proteins and is a major process for signal transduction and regulation of almost all biological functions. It is possible that members of the FIKK family are involved in mechanisms of sensing and/or protein transport to the red blood cell membrane. The question arises why the expansion of the single copy gene from a *Plasmodium* ancestor has occurred in *P. falciparum*. An explanation may come from the fact that this parasite has developed a unique cytoadhesion and antigenic variation system encoded by the *var* gene family that is absent in other malaria species, with the exception of the closely related *Plasmodium reichenowi* lineage, which, interestingly, also has an expanded FIKK family (six copies) (Schneider and Mercereau-Pujalon, 2005). It is likely that trafficking and correct exposure at the surface of the variant PfEMP1 and other molecules require the assistance of a number of other gene products. The interaction with variant surface molecules is further supported by the fact that all FIKK members have themselves a variable N-terminal region adjacent to the relatively conserved kinase catalytic domain. Besides the expansion of the FIKK kinase family, other single copy genes have been amplified from a *Plasmodium* ancestral gene such as the PHIST family (Sargeant *et al.*, 2006).

Our results suggest that these FIKK proteins may indeed function as kinases *in vivo*. A more unlikely possibility is that the observed activity was due to a co-precipitating kinase rather than to the FIKK itself. The role of phosphorylation in various stages of parasite development and infection of the host cell is well documented. It is known that phosphorylation and dephosphorylation processes play an important role during merozoite invasion of the erythrocytes (Rangachari *et al.*, 1986), during the life cycle of the parasite in the erythrocytes (Yuthavong and Limpaboon, 1987), in regulation of exflagellation of the male gametocyte (Billker *et al.*, 2004; Rangarajan *et al.*, 2005) and of ookinete formation during the sexual life stage of *Plasmodium berghei* (Reininger *et al.*, 2005). Infection of red blood cells by *P. falciparum* leads to a marked increase in the level of phosphorylation of some host erythrocytic membrane proteins, like protein 4.1 (Eder *et al.*, 1986) and to the insertion into the erythrocyte skeleton of parasite-encoded phosphoproteins. Comparing phosphorylation properties of knob-positive and knob-negative *Plasmodium*-infected erythrocytes, it was suggested that the ability to cytoadhere (displayed by knob-positive parasites) is linked with protein phosphorylation (Coppel *et al.*, 1988); furthermore, some patterns of inhibition of protein phosphorylation (Magowan *et al.*, 1998) are different between knob-positive and -negative parasites, suggesting a link between adhesion molecules and phosphorylation.

A large number of phosphoproteins have been demonstrated in the intraerythrocytic parasite (Wiser and Plitt,

1987; Jones and Edmundson, 1990), some of which are associated with the plasma membrane of the host cell (Coppel *et al.*, 1988; Foley *et al.*, 1990), such as RESA and MESA, two phosphoproteins thought to be involved in destabilization of the IE skeleton membrane. These proteins are phosphorylated in a developmental stage-specific manner (Jones and Edmundson, 1990). *P. falciparum* protein kinase activity appears to increase as the parasite progresses through the asexual replicative cycle in the erythrocyte (Wiser and Plitt, 1987). Like gene expression, kinase activity varies as a function of the parasitic stage. Whether or not the *Plasmodium* protein kinases regulate the expression of specific genes remains to be determined. An exciting possibility is that FIKK kinases may modify endogenous quiescent red blood cell proteins leading to the activation of channels in *P. falciparum*-infected erythrocytes as has been suggested recently (Bouyer *et al.*, 2006).

Previous studies have shown that several parasite protein kinases encoded by single copy genes are targeted to the Maurer's clefts or the tubovesicular system. These include *P. falciparum* exported serine–threonine kinase (Kun *et al.*, 1997), *P. falciparum* glycogen synthase kinase 3 (Droucheau *et al.*, 2004) and *P. falciparum* calcium-dependent protein kinase 1 (Moskes *et al.*, 2004). The localization of three FIKK proteins in the Maurer's clefts suggests that these structures are crucial in the trafficking of signalling pathway components.

Outside the catalytic domain, the FIKK proteins have no homology with regulatory domains of other protein kinases, indicating that they might be regulated by different signals from those modulating kinase activity in higher eukaryotes, or that they might be associated with other regulatory subunits. Alternative modes of regulation have been suggested previously for other *P. falciparum* kinases (Zhao *et al.*, 1992; Le Roch *et al.*, 2000). An interesting feature of the FIKK family members is that they are non-RD kinases. In general, kinase regulation can be achieved through a wide variety of mechanisms and many kinases rely on specific phosphorylation of regulatory domains. In many instances this is achieved by autophosphorylation of the activation loop, a process that is observed in the so-called RD kinases, which possess an arginine residue preceding the invariant aspartate required for catalytic activity (Johnson *et al.*, 1996). The FIKKs, like a minority of eukaryotic kinases, lack the conserved arginine (non-RD kinases) and probably do not autophosphorylate the activation loop; they can be predicted to be either constitutively active, or regulated through alternative mechanisms (Anamika *et al.*, 2005).

Real-time PCR transcription analysis of FIKK genes in *in vitro* cultured FCR3 parasites showed detectable transcription levels for all FIKK members. The steady-state transcript level, however, varies drastically between indi-

vidual members from two times superior to calmodulin to 10 times lesser than the reference gene (see Fig. 4). Furthermore, some *FIKK* gene transcripts are more abundant at the end of the cycle at the schizont stage, while others peak at ring stage. Unlike the expression of the *var* gene family, our data suggest that the subtelomeric *FIKK* genes are apparently not mutually exclusive transcribed. Taken together, the observed differences in the expression profile of the *FIKK* members most likely reflect the development of diverse functions after the expansion process of the primordial *FIKK* gene.

We observed that four members had very low levels of transcripts compared with our internal control calmodulin. These *FIKK* genes may be activated by internal stimuli such as changes in the adhesive properties of IEs. This is supported by our data showing that *FIKK7.1* transcription levels increased 3.5 times in FCR3CSA-selected parasites, while many other *FIKK* genes were slightly downregulated. Although the biological relevance remains unknown, it appears that the switch in expression to CSA-binding parasites involves a general change in the transcription pattern of several genes. For example, the *MESA* gene, which codes for a secreted protein that associates with the erythrocyte membrane, is also downregulated in FCR3CSA parasites (Ralph *et al.*, 2005). It will be interesting to investigate whether these changes are specific adaptations of the parasite to the expression of variant surface molecules.

A recent microarray study by Daily *et al.* (2005) showed that steady-state mRNA levels for nine *FIKK* genes was increased by a factor of at least two in a patient isolate, compared with *in vitro* 3D7 samples. This suggests that external factors found in the serum may influence the transcription pattern of *FIKK* genes. It will be important to use parasites from pregnant women to determine if a particular *FIKK* member is upregulated during pregnancy, possibly due to particular hormones only present during pregnancy.

In conclusion, we show that members of the *FIKK* kinase family are exported into the cytoplasm of the IE to distinct locations, and that at least some of these molecules are associated with protein kinase activity. This supports that particular members may have evolved different functions and it will be interesting to study their role in the remodelling of the red blood cell membrane.

## Experimental procedures

### *Plasmodium falciparum* cultures

*P. falciparum* blood stage parasites from the 3D7 and FCR3 strains were cultured using modifications to the method described by Trager and Jensen (1976). Parasites were grown in O+ human erythrocytes in RPMI 1640 medium containing L-glutamine (Invitrogen) supplemented with 5%

v/v human serum (PAA Laboratories GmbH) and 5% v/v Albumax II (Invitrogen) in a gas environment of 5% CO<sub>2</sub>, 1% O<sub>2</sub> and 94% N<sub>2</sub>. Knob-positive parasites were routinely selected by gelatin flotation using plasmin (Fresenius Kabi) (Lelievre *et al.*, 2005). Synchronization of cultures consisted of two consecutive 5% sorbitol (Sigma) treatments (Lambros and Vanderberg, 1979). We estimated that the parasites were synchronized within a window of approximately 6 h by giemsa staining.

### Panning of *P. falciparum*-infected erythrocytes

*P. falciparum* strain FCR3 was selected on the human placental derived BeWo cell line (European Collection of Cell Cultures) to obtain the FCR3CSA parasite line (Viebig *et al.*, 2006) or on Chinese Hamster Ovary cells PgsA-745 (CHO-745) (American Type Culture Collection) to obtain the FCR3CD36 parasite line. Pannings were repeated twice more, and parasites were tested for their ability to bind purified CSA (Sigma) or soluble recombinant CD36 (Affymax Research Institute) immobilized with monoclonal antibody 179 (Affymax Research Institute) (Baruch *et al.*, 1996).

### Antibodies

To obtain polyclonal antibody serum against PFD1165w (*FIKK4.1*), PFI0105c (*FIKK9.3*) and PFI0120c (*FIKK9.6*) we used the Eurogentec standard protocols (<http://www.eurogentec.com>) for immunizing guinea pigs and rats. Each animal was immunized with a combination of two synthetic peptides. The peptide sequences are as follows: *FIKK4.1*, H2N-RRSQEVFKTEVSKD[+C]-CONH2 and H2N-RHLKGNKRLRYYESY[+C]-CONH2; *FIKK9.3*, H2N-CTNDYLGTTTDDSTKF-CONH2 and H2N-LNKVPTKNYSERSGR[+C]-CONH2; *FIKK9.6*, H2N-NTYDEYESYKRIKK[+C]-CONH2 and H2N-CFRDRYYNERKGRVHK-CONH2. To obtain IgYs against PFL0040c (*FIKK12*) and PFI0100c (*FIKK9.2*), synthetic peptides H2N-RFTKEKSDVRNSDEF[+C]-CONH2 and H2N-KNENYNESGGRNHEM[+C]-CONH2 derived from the protein sequences (residues 331–345 and 161–175 respectively) were used for immunization of chickens. The additional C-terminal Cys residues allowed coupling to rabbit albumin (Goldring and Coetzer, 2003). Protocols for immunization, isolation and affinity purification of IgYs, were described previously (Merckx *et al.*, 2003).

### Immunofluorescence microscopy

Synchronized IEs were washed in phosphate-buffered saline (PBS) (0.15 M NaCl and 10 mM sodium phosphate, pH 7.2). Cell pellets were resuspended in 5 vols of PBS and a monolayer was set onto microscope slides. Parasites were air-dried and fixed for 10 min at room temperature in 2% paraformaldehyde. Slides were blocked for 20 min in 0.1% bovine serum albumin (BSA) and incubated with the primary antibody diluted in 0.1% BSA for 45 min at room temperature. After washing cells were incubated for 30 min with a secondary antibody conjugated with fluorochrome (Invitrogen) in a 1:500 dilution. Slides were washed thoroughly in PBS and mounted in Vectashield anti-fading with 4-6-diamidino-2-

phenylindole (DAPI; Vector Laboratories). Images were captured using a Nikon Elipse 80i optical microscope.

Anti-FlkK sera were pre-absorbed with lysed erythrocytes before incubation with the IEs. The final antibody dilutions were: guinea pig anti-FlkK4.1 1:100, guinea pig anti-FlkK9.3 1:60, rat anti-FlkK9.6 1:250, mouse anti-SBP1 1:500, mouse anti-HSP70 1:800. The chicken antibodies were used at 2 µg ml<sup>-1</sup> (anti-FlkK9.2) and 0.5 µg ml<sup>-1</sup> (anti-FlkK12).

Peptide competition assays were performed to test the specificity of the anti-FlkK sera. Increasing concentrations of the specific and unspecific peptides were added to the serum and incubated overnight in a rotating wheel at 4°C.

To perform indirect immunofluorescence in the GFP-transfected parasites we used the method described by Tonkin *et al.* (2004). Briefly IEs were washed in PBS, fixed in suspension with 4% paraformaldehyde containing 0.0075% glutaraldehyde, permeabilized in 0.1% Triton X-100, treated with 0.1 mg ml<sup>-1</sup> of sodium borohydride and blocked in 3% BSA. Cells were then sequentially incubated with anti-FlkK12 antibody and goat anti-chicken secondary antibody. After the final washes cells were settled on coverslips coated with 1% polyethylenimine (Sigma).

#### Plasmid construct and parasite transfection

DNA-free RNA was reverse transcribed with a specific primer for the 3' UTR region of the FlkK12: 5'-AAAAAAA CATATTAATAACAATCATAC-3'. FlkK12 gene was amplified using Expand High Fidelity PCR System (Roche) with primers containing introduced restriction sites for the enzymes AvrII and XhoI, forward primer 5'-GGAGCTC GAGATGTATATTTTGGAGAAATATGTTCTG-3', reverse primer 5'-ACTGCC TAGGTGTTTCGTTAAACCATGGGTGTG-3' (enzyme restriction sites in italics) and cloned in frame with the GFP tag into the plasmid vector pARL (Crabb *et al.*, 2004).

3D7 ring stage parasites were transfected by electroporation and drug selected using 0.25 mM of the anti-folate drug WR99210 (Fidock and Wellem, 1997).

#### Immunoprecipitation and kinase assay

Infected erythrocytes enriched by plasmion flotation or uninfected erythrocytes were solubilized in lysis buffer [20 mM Tris/HCl pH 8, 10 mM EDTA, 400 mM NaCl, 3% Triton X-100, protease inhibitor cocktail tablets, 1 mM phenylmethanesulphonyl fluoride (PMSF), 10 mM sodium fluoride (NaF), 10 mM glycerophosphate]. After sonication and centrifugation the lysates were pre-cleared by incubation with protein A Sepharose CL-4B (Amersham) or G Sepharose (Upstate) for 1 h. After the extracts were incubated with either the polyclonal serum (15 µl), the pre-immune serum, anti-FlkK12 (2.5 µg) or anti-GFP (2 µg) for 2 h at 4°C. Immunoprecipitates were collected with protein A Sepharose, protein A Sepharose coated with rabbit anti-chicken IgY (when using chicken anti-FlkK12) (Merck *et al.*, 2003) or G Sepharose (when using mouse anti-GFP) and extensively washed. Before kinase assay the immunocomplexes were washed with kinase assay buffer (25 mM Tris/HCl pH 8, 20 mM MgCl<sub>2</sub>, 2 mM MnCl<sub>2</sub>, protease inhibitor, 1 mM PMSF, 10 mM NaF, 10 mM glycerophosphate).

The immunocomplexes were then resuspended in a final volume of 30 µl of kinase assay buffer with or without 5 µg of MBP. The reaction was carried out in the presence of 25 µM ATP and 5 µCi of [<sup>32</sup>P]-ATP (6000 Ci mmol<sup>-1</sup>; MP Biomedicals) at 30°C for 30 min. After incubation the reaction was stopped by adding Laemmli sample buffer and boiled for 5 min. Samples were analysed by 12% SDS-PAGE and then autoradiography.

#### RNA preparation and real-time PCR

Synchronized IEs were washed in PBS, permeabilized with 0.05% saponin and preserved in Trizol (Invitrogen) at -80°C until isolation of RNA. Total RNA was prepared following the protocol by Kyes *et al.* (2000). RNA aliquots were treated with DNA-free kit (Ambion) to degrade contaminating genomic DNA. DNA-free RNA was reverse transcribed with SuperscriptIII Rnase H Reverse Transcriptase (Invitrogen) and primed with oligo-dT primers (Invitrogen). For each cDNA synthesis reaction a control reaction without reverse transcriptase was performed.

Quantitative real-time PCR was done in a Realplex4 EppgradientS thermal cycler (Eppendorf), amplification products were designed to be less than 200 bp in size, the *P. falciparum calmodulin* gene (*PF14\_0323*) and *seryl-tRNA synthetase* gene (*PF07\_0073*) were used as internal controls. Reactions were performed in 20 µl volumes using SYBR Green PCR master mix (Applied Biosystems) and 400 µM primers, according to manufacturer's instruction. PCR cycling condition were: 94°C for 10 min followed by 40 cycles of 94°C/15 s 50°C/30 s and 62°C/30 s. Data were acquired at the end of the elongation step of each cycle. Melting-curve analysis was done at the end of each program to prove specificity of the amplification; specificity was further ascertained by a single band on ethidium bromide stained gel. Water and no-reverse transcriptase controls were included in each run. Each gene was analysed in duplicate using RNA from two or three independent RNA isolations. Standard curves with serial dilutions of genomic DNA (five dilutions over a 1000-fold concentration range) were included with each PCR run. Transcription level of each gene was assessed as relative copy number to the housekeeping gene *calmodulin* using the equation obtained through the standard curve. The gene *PFC0060c* was not detectable in our FCR3 strain, this gene was not included in our study. The pairs of primers used are listed in Table S1.

#### Ghost preparation

Erythrocyte ghosts were prepared from trophozoite-infected erythrocytes as described previously (Blisnick *et al.*, 2000). Briefly, synchronized IEs were extensively washed in RPMI 1640 medium and lysed for 15 min at 4°C in 40 vols of hypotonic buffer (RPMI 1640 diluted 1/5 in water with protease inhibitors). The lysates were then separated by centrifugation into a cytosolic fraction and a pellet containing ghosts and free parasites. The erythrocyte ghosts were collected at the top of the free parasite pellet, washed extensively in the hypotonic buffer, then incubated in PBS for 30 min at 37°C and further handled for indirect immunofluorescence.

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### References

- Anamika, Srinivasan, N., and Krupa, A. (2005) A genomic perspective of protein kinases in *Plasmodium falciparum*. *Proteins* **58**: 180–189.
- Barnwell, J.W., Howard, R.J., Coon, H.G., and Miller, L.H. (1983) Splenic requirement for antigenic variation and expression of the variant antigen on the erythrocyte membrane in cloned *Plasmodium knowlesi* malaria. *Infect Immun* **40**: 985–994.
- Baruch, D.I., Pasloske, B.L., Singh, H.B., Bi, X., Ma, X.C., Feldman, M., *et al.* (1995) Cloning the *P. falciparum* gene encoding PfEMP1, a malarial variant antigen and adherence receptor on the surface of parasitized human erythrocytes. *Cell* **82**: 77–87.
- Baruch, D.I., Gormely, J.A., Ma, C., Howard, R.J., and Pasloske, B.L. (1996) *Plasmodium falciparum* erythrocyte membrane protein 1 is a parasitized erythrocyte receptor for adherence to CD36, thrombospondin, and intercellular adhesion molecule 1. *Proc Natl Acad Sci USA* **93**: 3497–3502.
- Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* **117**: 503–514.
- Blisnick, T., Morales Betoulle, M.E., Barale, J.C., Uzureau, P., Berry, L., Desroses, S., *et al.* (2000) Pfsbp1, a Maurer's cleft *Plasmodium falciparum* protein, is associated with the erythrocyte skeleton. *Mol Biochem Parasitol* **111**: 107–121.
- Bonnefoy, S., Guillotte, M., Langsley, G., and Mercereau-Pujalon, O. (1992) *Plasmodium falciparum*: characterization of gene R45 encoding a trophozoite antigen containing a central block of six amino acid repeats. *Exp Parasitol* **74**: 441–451.
- Bouyer, G., Egee, S., and Thomas, S.L. (2006) Three types of spontaneously active anionic channels in malaria-infected human red blood cells. *Blood Cells Mol Dis* **36**: 248–254.
- Brown, K.N. (1973) Antibody induced variation in malaria parasites. *Nature* **242**: 49–50.
- Coppel, R.L., Lustigman, S., Murray, L., and Anders, R.F. (1988) MESA is a *Plasmodium falciparum* phosphoprotein associated with the erythrocyte membrane skeleton. *Mol Biochem Parasitol* **31**: 223–231.
- Crabb, B.S., Rug, M., Gilberger, T.W., Thompson, J.K., Triglia, T., Maier, A.G., and Cowman, A.F. (2004) Transfection of the human malaria parasite *Plasmodium falciparum*. *Methods Mol Biol* **270**: 263–276.
- Daily, J.P., Le Roch, K.G., Sarr, O., Ndiaye, D., Lukens, A., Zhou, Y., *et al.* (2005) In vivo transcriptome of *Plasmodium falciparum* reveals overexpression of transcripts that encode surface proteins. *J Infect Dis* **191**: 1196–1203.
- David, P.H., Hommel, M., Miller, L.H., Udeinya, I.J., and Oligino, L.D. (1983) Parasite sequestration in *Plasmodium falciparum* malaria: spleen and antibody modulation of cytoadherence of infected erythrocytes. *Proc Natl Acad Sci USA* **80**: 5075–5079.
- Dorin, D., Semblat, J.P., Pouillet, P., Alano, P., Goldring, J.P., Whittle, C., *et al.* (2005) PfPK7, an atypical MEK-related protein kinase, reflects the absence of classical three-component MAPK pathways in the human malaria parasite *Plasmodium falciparum*. *Mol Microbiol* **55**: 184–196.
- Droucheau, E., Primot, A., Thomas, V., Mattei, D., Knockaert, M., Richardson, C., *et al.* (2004) *Plasmodium falciparum* glycogen synthase kinase-3: molecular model, expression, intracellular localisation and selective inhibitors. *Biochim Biophys Acta* **1697**: 181–196.
- Eder, P.S., Soong, C.J., and Tao, M. (1986) Phosphorylation reduces the affinity of protein 4.1 for spectrin. *Biochemistry* **25**: 1764–1770.
- Fidock, D.A., and Wellems, T.E. (1997) Transformation with human dihydrofolate reductase renders malaria parasites insensitive to WR99210 but does not affect the intrinsic activity of proguanil. *Proc Natl Acad Sci USA* **94**: 10931–10936.
- Foley, M., Murray, L.J., and Anders, R.F. (1990) The ring-infected erythrocyte surface antigen protein of *Plasmodium falciparum* is phosphorylated upon association with the host cell membrane. *Mol Biochem Parasitol* **38**: 69–75.
- Freitas-Junior, L.H., Bottius, E., Pirit, L.A., Deitsch, K.W., Scheidig, C., Guinet, F., *et al.* (2000) Frequent ectopic recombination of virulence factor genes in telomeric chromosome clusters of *P. falciparum*. *Nature* **407**: 1018–1022.
- Fried, M., and Duffy, P.E. (1996) Adherence of *Plasmodium falciparum* to chondroitin sulfate A in the human placenta. *Science* **272**: 1502–1504.
- Gamain, B., Trimnell, A.R., Scheidig, C., Scherf, A., Miller, L.H., and Smith, J.D. (2005) Identification of multiple chondroitin sulfate A (CSA)-binding domains in the *var2CSA* gene transcribed in CSA-binding parasites. *J Infect Dis* **191**: 1010–1013.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., *et al.* (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511.
- Goldring, J.P.D., and Coetzer, T.H.T. (2003) Isolation of chicken immunoglobulins (IgY) from egg yolk. *Biochem Mol Biol Edu* **31**: 185–187.
- Hanks, S.K. (2003) Genomic analysis of the eukaryotic protein kinase superfamily: a perspective. *Genome Biol* **4**: 111.
- Hanks, S.K., Quinn, A.M., and Hunter, T. (1988) The protein kinase family: conserved features and deduced phylogeny of the catalytic domains. *Science* **241**: 42–52.

- Hiller, N.L., Bhattacharjee, S., van Ooij, C., Liolios, K., Harrison, T., Lopez-Estrano, C., and Haldar, K. (2004) A host-targeting signal in virulence proteins reveals a secretome in malarial infection. *Science* **306**: 1934–1937.
- Hinterberg, K., Scherf, A., Gysin, J., Toyoshima, T., Aikawa, M., Mazie, J.C., *et al.* (1994) *Plasmodium falciparum*: the Pf332 antigen is secreted from the parasite by a brefeldin A-dependent pathway and is translocated to the erythrocyte membrane via the Maurer's clefts. *Exp Parasitol* **79**: 279–291.
- Johnson, L.N., Noble, M.E., and Owen, D.J. (1996) Active and inactive protein kinases: structural basis for regulation. *Cell* **85**: 149–158.
- Jones, G.L., and Edmundson, H.M. (1990) Protein phosphorylation during the asexual life cycle of the human malarial parasite *Plasmodium falciparum*. *Biochim Biophys Acta* **1053**: 118–124.
- Knighton, D.R., Zheng, J.H., Ten Eyck, L.F., Ashford, V.A., Xuong, N.H., Taylor, S.S., and Sowadski, J.M. (1991) Crystal structure of the catalytic subunit of cyclic adenosine monophosphate-dependent protein kinase. *Science* **253**: 407–414.
- Kooij, T.W., Carlton, J.M., Bidwell, S.L., Hall, N., Ramesar, J., Janse, C.J., and Waters, A.P. (2005) A plasmodium whole-genome synteny map: indels and synteny breakpoints as foci for species-specific genes. *PLoS Pathog* **1**: e44.
- Kun, J.F., Hibbs, A.R., Saul, A., McColl, D.J., Coppel, R.L., and Anders, R.F. (1997) A putative *Plasmodium falciparum* exported serine/threonine protein kinase. *Mol Biochem Parasitol* **85**: 41–51.
- Kyes, S., Pinches, R., and Newbold, C. (2000) A simple RNA analysis method shows *var* and *rif* multigene family expression patterns in *Plasmodium falciparum*. *Mol Biochem Parasitol* **105**: 311–315.
- Kyes, S., Horrocks, P., and Newbold, C. (2001) Antigenic variation at the infected red cell surface in malaria. *Annu Rev Microbiol* **55**: 673–707.
- Lambros, C., and Vanderberg, J.P. (1979) Synchronization of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* **65**: 418–420.
- Le Roch, K., Sestier, C., Dorin, D., Waters, N., Kappes, B., Chakrabarti, D., *et al.* (2000) Activation of a *Plasmodium falciparum* cdc2-related kinase by heterologous p25 and cyclin H. Functional characterization of a *P. falciparum* cyclin homologue. *J Biol Chem* **275**: 8952–8958.
- Lelievre, J., Berry, A., and Benoit-Vical, F. (2005) An alternative method for *Plasmodium* culture synchronization. *Exp Parasitol* **109**: 195–197.
- Magowan, C., Liang, J., Yeung, J., Takakuwa, Y., Coppel, R.L., and Mohandas, N. (1998) *Plasmodium falciparum*: influence of malarial and host erythrocyte skeletal protein interactions on phosphorylation in infected erythrocytes. *Exp Parasitol* **89**: 40–49.
- Marti, M., Good, R.T., Rug, M., Knuepfer, E., and Cowman, A.F. (2004) Targeting malaria virulence and remodeling proteins to the host erythrocyte. *Science* **306**: 1930–1933.
- Mattei, D., Scherf, A., Bensaude, O., and da Silva, L.P. (1989) A heat shock-like protein from the human malaria parasite *Plasmodium falciparum* induces autoantibodies. *Eur J Immunol* **19**: 1823–1828.
- Merckx, A., Le Roch, K., Nivez, M.P., Dorin, D., Alano, P., Gutierrez, G.J., *et al.* (2003) Identification and initial characterization of three novel cyclin-related proteins of the human malaria parasite *Plasmodium falciparum*. *J Biol Chem* **278**: 39839–39850.
- Moskes, C., Burghaus, P.A., Wernli, B., Sauder, U., Durrenberger, M., and Kappes, B. (2004) Export of *Plasmodium falciparum* calcium-dependent protein kinase 1 to the parasitophorous vacuole is dependent on three N-terminal membrane anchor motifs. *Mol Microbiol* **54**: 676–691.
- Ralph, S.A., Bischoff, E., Mattei, D., Sismeiro, O., Dillies, M.A., Guigon, G., *et al.* (2005) Transcriptome analysis of antigenic variation in *Plasmodium falciparum* – var silencing is not dependent on antisense RNA. *Genome Biol* **6**: R93.
- Rangachari, K., Dluzewski, A., Wilson, R.J., and Gratzer, W.B. (1986) Control of malarial invasion by phosphorylation of the host cell membrane cytoskeleton. *Nature* **324**: 364–365.
- Rangarajan, R., Bei, A.K., Jethwaney, D., Maldonado, P., Dorin, D., Sultan, A.A., and Doerig, C. (2005) A mitogen-activated protein kinase regulates male gametogenesis and transmission of the malaria parasite *Plasmodium berghei*. *EMBO Rep* **6**: 464–469.
- Reininger, L., Billker, O., Tewari, R., Mukhopadhyay, A., Fennell, C., Dorin-Semblat, D., *et al.* (2005) A NIMA-related protein kinase is essential for completion of the sexual cycle of malaria parasites. *J Biol Chem* **280**: 31957–31964.
- Salanti, A., Staalsoe, T., Lavstsen, T., Jensen, A.T., Sowa, M.P., Arnot, D.E., *et al.* (2003) Selective upregulation of a single distinctly structured *var* gene in chondroitin sulphate A-adhering *Plasmodium falciparum* involved in pregnancy-associated malaria. *Mol Microbiol* **49**: 179–191.
- Sargeant, T.J., Marti, M., Caler, E., Carlton, J.M., Simpson, K., Speed, T.P., and Cowman, A.F. (2006) Lineage-specific expansion of proteins exported to erythrocytes in malaria parasites. *Genome Biol* **7**: R12.
- Scherf, A., Hernandez-Rivas, R., Buffet, P., Bottius, E., Benatar, C., Pouvelle, B., *et al.* (1998) Antigenic variation in malaria: in situ switching, relaxed and mutually exclusive transcription of *var* genes during intra-erythrocytic development in *Plasmodium falciparum*. *EMBO J* **17**: 5418–5426.
- Schneider, A.G., and Mercereau-Pujalon, O. (2005) A new *Apicomplexa*-specific protein kinase family: multiple members in *Plasmodium falciparum*, all with an export signature. *BMC Genomics* **6**: 30.
- Smith, J.D., Chitnis, C.E., Craig, A.G., Roberts, D.J., Hudson-Taylor, D.E., Peterson, D.S., *et al.* (1995) Switches in expression of *Plasmodium falciparum* *var* genes correlate with changes in antigenic and cytoadherent phenotypes of infected erythrocytes. *Cell* **82**: 101–110.
- Stanley, H.A., Langreth, S.G., and Reese, R.T. (1989) *Plasmodium falciparum* antigens associated with membrane structures in the host erythrocyte cytoplasm. *Mol Biochem Parasitol* **36**: 139–149.
- Su, X.Z., Heatwole, V.M., Wertheimer, S.P., Guinet, F., Herfeldt, J.A., Peterson, D.S., *et al.* (1995) The large diverse gene family *var* encodes proteins involved in cytoadherence and antigenic variation of *Plasmodium falciparum*-infected erythrocytes. *Cell* **82**: 89–100.

- Tonkin, C.J., van Dooren, G.G., Spurck, T.P., Struck, N.S., Good, R.T., Handman, E., *et al.* (2004) Localization of organellar proteins in *Plasmodium falciparum* using a novel set of transfection vectors and a new immunofluorescence fixation method. *Mol Biochem Parasitol* **137**: 13–21.
- Trager, W., and Jensen, J.B. (1976) Human malaria parasites in continuous culture. *Science* **193**: 673–675.
- Viebig, N.K., Gamain, B., Scheidig, C., Lepolard, C., Przyborski, J., Lanzer, M., *et al.* (2005) A single member of the *Plasmodium falciparum* var multigene family determines cytoadhesion to the placental receptor chondroitin sulphate A. *EMBO Rep* **6**: 775–781.
- Viebig, N.K., Nunes, M.C., Scherf, A., and Gamain, B. (2006) The human placental derived BeWo cell line: a useful model for selecting *Plasmodium falciparum* CSA-binding parasites. *Exp Parasitol* **112**: 121–125.
- Ward, P., Equinet, L., Packer, J., and Doerig, C. (2004) Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. *BMC Genomics* **5**: 79.
- Wiser, M.F., and Plitt, B. (1987) *Plasmodium berghei*, *P. chabaudi*, and *P. falciparum*: similarities in phosphoproteins and protein kinase activities and their stage specific expression. *Exp Parasitol* **64**: 328–335.
- Yuthavong, Y., and Limpiboon, T. (1987) The relationship of phosphorylation of membrane proteins with the osmotic fragility and filterability of *Plasmodium berghei*-infected mouse erythrocytes. *Biochim Biophys Acta* **929**: 278–287.
- Zhao, Y., Kappes, B., Yang, J., and Franklin, R.M. (1992) Molecular cloning, stage-specific expression and cellular

distribution of a putative protein kinase from *Plasmodium falciparum*. *Eur J Biochem* **207**: 305–313.

### Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** z stacking image of FIKK12-GFP expressing parasite. Serial images were acquired at 0.35  $\mu\text{m}$  intervals showing the GFP signal (green) at the periphery of the infected red blood cell. Nuclei of *P. falciparum* detected by DAPI staining (blue).

**Fig. S2.** Peptide competition assay. The specificity of FIKK4.1 (A) and FIKK9.3 (B) sera was determined by IFA after pre-incubating the antibodies with increasing concentrations of the specific FIKK peptide used to raise the antibodies or with unspecific peptides. The decrease in the antibody signal is striking using increased concentrations of the specific peptides but it is not affected by the incubation with the unspecific peptides.

**Fig. S3.** Real-time PCR analysis of transcription levels of the entire FIKK family in FCR3CSA- and FCR3CD36-infected erythrocytes. FCR3CSA- and FCR3CD36-selected parasites RNA was extracted at three different time points of the intraerythrocytic cycle. All values represent the mean of two or three independent RNA isolations  $\pm$  SD as relative copy number to the housekeeping gene *calmodulin*.

**Table S1.** List of pair of primers used in real-time PCR analysis.

This material is available as part of the online article from <http://www.blackwell-synergy.com>

## Theoretical epidemiology

During the year that I was a research scientist at the Theoretical Epidemiology Group, at the Instituto Gulbenkian de Ciência in Oeiras, Portugal, I participated in two main projects: the first one aimed at developing mathematical models of the complex mechanism of antigenic variation in *Plasmodium falciparum* and in the second project we used data from molecular epidemiology studies to discriminate between relapses of latent tuberculosis infection or exogenous reinfection in recurrent episodes of tuberculosis.

With my background on malaria research I helped to develop new concepts of antigenic variation and host-parasite interactions. We used available molecular and serological data on *Plasmodium falciparum* variant surface antigens to formulate a mathematical model of the evolutionary mechanisms shaping these antigens' organization and expression patterns. The model was able to reproduce immunological trends observed in field data, and identified the mechanisms that might be driving the evolution of separate clusters of variant surface antigens in *P. falciparum*. Our results predicted the existence of a restricted subset of high-dominance variant surface antigens associated with severe malaria, and genetically and immunologically diverse low-dominance antigens related to uncomplicated and asymptomatic infection. I co-authored a manuscript describing these findings (Van Noort et al. 2010).

The tuberculosis research resulted in a manuscript where we proposed a minimal model for tuberculosis transmission to describe the relative contributions of reinfection and relapse to recurrent tuberculosis across a range of transmission intensities that could be used for control planning (Gomes et al. 2012).

## Vaccine preventable diseases

At the Vaccines & Infectious Diseases Analytics (Wits VIDA) Research Unit in South Africa my research was focused on reducing under-5 childhood morbidity and mortality from leading infectious causes. This included evaluation of pneumococcal conjugate-vaccines in a setting with high Human Immunodeficiency Virus (HIV) burden, clinical and molecular epidemiology of respiratory viruses associated with pneumonia and immunization of pregnant women. In a large multi-year surveillance study, I investigated the relevance of *Bordetella pertussis*, Influenza virus and Respiratory Syncytial Virus infections (RSV) in hospitalized patients; with the aim to describe the burden of these pathogens and clarify the need for future vaccines. To approach the problem of infant morbidity related to infections from a different angle I explored the potential of intervening through vaccination of pregnant women to protect the women and their babies against infections. After conducting two randomized-placebo-control trials of maternal influenza vaccination I led a large case-control study on the effectiveness of maternal influenza vaccination against hospitalizations. Currently I am still the principal investigator of an immunogenicity study of pertussis vaccination comparing the humoral response between HIV-uninfected pregnant women and pregnant women living with HIV.

My research portfolio at Wits VIDA can be divided into four broad topics that will be illustrated below by key projects and publications.

1. Evaluation of pneumococcal conjugate vaccines
2. Epidemiology of respiratory infections, including surveillance studies
3. Maternal immunization, including women living with HIV
4. COVID-19



## Evaluation of pneumococcal conjugate vaccines

I performed analyses on the national South African database for invasive pneumococcal disease (IPD) surveillance to establish baseline information on the risk of IPD before pneumococcal conjugate-vaccine (PCV) introduction in South Africa and to provide guidance on the IPD risk-reduction due to antiretroviral therapy that needed to be accounted for when vaccine impact studies were performed in HIV-infected children (Marta C Nunes et al. 2011) and adults (Marta C. Nunes et al. 2011).

In South Africa, 7-valent PCV (PCV7) was first introduced in April 2009 as a 2+1 schedule for infants at 6 weeks, 14 weeks, and 9 months of age. PCV7 was replaced with 13-valent PCV (PCV13) in April 2011, with gradual upscaling of coverage by the end of 2011. I investigated pneumococcal colonization acquisition in children who received PCV at 6, 14 and 40 weeks age (the vaccination schedule implemented in South Africa) and compared it to cohorts of PCV-naïve children and children who received a 3+1 schedule (the WHO recommended schedule) (Marta C. Nunes et al. 2013; Marta C. Nunes, Jones, et al. 2015; Marta Coelho Nunes et al. 2020). While PCV7 was introduced with no catch-up strategy implemented, a catch-up program was recommended following transition to PCV13. This catch-up included all children between 18 and 36 months of age, and children 36 and 71 months of age with underlying medical conditions, including immunocompromising conditions and chronic diseases. In a clinical study that I led we investigated the immunogenicity of PCV13 among children with underlying medical conditions, including HIV-infection and those with kidney and lung diseases (at-risk children), and a healthy control group. We found that all study-groups mounted an immune response to PCV13, with the at-risk groups having responses that were mostly similar to the control children (Jallow et al. 2017).

Employing a vaccine probe design approach, I used data collected during a historical placebo-controlled randomized clinical trial assessing the effectiveness of an experimental 9-valent PCV (PCV9) against IPD, to investigate the impact of this vaccine on viral infections. We demonstrated that children who received PCV9 had a lower risk of polyomaviruses-associated respiratory infections (Marta C. Nunes et al. 2014) and seasonal coronaviruses pneumonia hospitalizations (Marta C. Nunes et al. 2021) demonstrating an impact of vaccination beyond pneumococcal prevention.

Attached four papers related to this topic.

# Persistent High Burden of Invasive Pneumococcal Disease in South African HIV-Infected Adults in the Era of an Antiretroviral Treatment Program

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## Abstract

**Background:** Highly active antiretroviral treatment (HAART) programs have been associated with declines in the burden of invasive pneumococcal disease (IPD) in industrialized countries. The aim of this study was to evaluate trends in IPD hospitalizations in HIV-infected adults in Soweto, South Africa, associated with up-scaling of the HAART program from 2003 to 2008.

**Methods:** Laboratory-confirmed IPD cases were identified from 2003 through 2008 through an existing surveillance program. The period 2003–04 was designated as the early-HAART era, 2005–06 as the intermediate-HAART era and 2007–08 as the established-HAART era. The incidence of IPD was compared between the early-HAART and established-HAART eras in HIV-infected and uninfected individuals.

**Results:** A total of 2,567 IPD cases among individuals older than 18 years were reported from 2003 through 2008. Overall incidence of IPD (per 100,000) did not change during the study period in HIV-infected adults (207.4 cases in the early-HAART and 214.0 cases in the established-HAART era;  $p=0.55$ ). IPD incidence, actually increased 1.16-fold (95% CI: 1.01; 1.62) in HIV-infected females between the early and established-HAART eras (212.1 cases and 246.2 cases, respectively;  $p=0.03$ ). The incidence of IPD remained unchanged in HIV-uninfected adults across the three time periods.

**Conclusion:** Despite a stable prevalence of HIV and the increased roll-out of HAART for treatment of AIDS patients in our setting, the burden of IPD has not decreased among HIV-infected adults. The study indicates a need for ongoing monitoring of disease and HAART program effectiveness to reduce opportunistic infections in African adults with HIV/AIDS, as well as the need to consider alternate strategies including pneumococcal conjugate vaccine immunization for the prevention of IPD in HIV-infected adults.

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**Competing Interests:** The authors have read the journal's policy and have the following conflicts: Receipt of research funding (SAM, AvG, KPK) and consultancy from Pfizer (SAM, KPK). Receipt of research funding (SAM, AvG, KPK), consultancies and honoraria from GSK (SAM, KPK). Consultancy for Merck (KPK). This does not alter the authors' adherence to all the PLoS ONE policies on sharing data and materials.

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## Introduction

HIV-infected adults in the absence of highly active antiretroviral treatment (HAART) have an increased risk of developing invasive disease from *Streptococcus pneumoniae* (IPD) and are at heightened susceptibility of recurrent IPD episodes. [1,2,3,4] The initiation of HAART in many developed countries since the mid-1990s has been associated with marked reductions in morbidity and

mortality from opportunistic infections due to humoral and cell-mediated immune reconstitution with HAART. [5] Protection against IPD is dependent upon opsonisation of the pneumococcus by antibodies and subsequent killing thereof by phagocytic activity. Two- to three-fold reductions in the risk of IPD have been observed among HIV-infected adults developed country settings where HAART programs have been effectively implemented. [6,7,8] Nevertheless, in the United States the incidence of IPD in

HIV-infected adults in the era of HAART continued to be approximately 35-fold greater than the general population. [7] The widespread use of HAART in industrializing countries has lagged behind that in developed countries so that the impact thereof on the burden of opportunistic infections remains to be fully quantified. Only a few studies have been published to date from Africa, the continent with the highest prevalence and number of individuals with HIV/AIDS, suggesting a reduction in opportunistic infections incidence with HAART usage. [9,10]

Following global initiatives for the up-scaling of HAART access for HIV-infected individuals in industrializing countries since 2003, South Africa embarked on a national program of HAART management in 2004. [11] The estimated prevalence of HIV among African adults aged 18–64 years was 25% in 2004 and 27% in 2008 in the province of Gauteng, South Africa, where this study was undertaken. [12,13,14] Previous studies on the impact of HIV/AIDS on IPD in adults in South Africa were undertaken prior to the HAART era. [2,15,16] Determination of the impact of HAART on the burden of IPD in African settings is necessary to determine whether the prevention of IPD in African HIV-infected individuals remains a priority.

The aim of this study was to evaluate the impact of the HAART program on the burden of hospitalization for IPD in South African HIV-infected adults.

## Methods

### Study design and study population

The analysis included all laboratory-confirmed IPD episodes hospitalized at Chris Hani-Baragwanath Hospital (CHBH) from January 2003 to December 2008. CHBH is a public hospital, providing curative health-care to approximately 1.4 million African urban South Africans living in Soweto. [17] Approximately 18% of the Sowetan population has private medical insurance and may attend private hospitals, whilst the remainder of the population among whom unemployment rate is 25% are primarily admitted to CHBH when requiring hospitalization. [17] Neither pneumococcal polysaccharide vaccine (PPV23) immunization of HIV-infected adults nor pneumococcal conjugate vaccine (PCV) immunization of children was available as standard-of-care during the period reviewed.

The analysis was divided into 3 eras according to the national estimates of HAART coverage of adults with AIDS. [18] The numerators for era-specific disease incidences were the numbers of IPD episodes hospitalized during each era, and the population denominator was based on the Gauteng Department of Health and Social Development, Statistics South Africa, population estimates for Region D (i.e. Soweto) in Johannesburg, South Africa. [17] HIV prevalence in the study population was estimated from the 2010 projections of the AIDS and Demographic model developed by the Actuarial Society of South Africa. [14] These estimates have been shown to correlate with estimates from population-based studies. [13] Exact figures of the number of HIV infected individuals requiring HAART treatment are difficult to estimate due to uncertainty about the number who fulfil the eligibility criteria for treatment. South African Department of Health criteria for HAART initiation in adolescents and adults during the period under review were: CD4+ T lymphocyte (CD4+) cell count less than 200 cells per microliter; or World Health Organization (WHO) stage IV irrespective of CD4+ cell count. [19] Considering these criteria and the prevalence of HIV/AIDS, the national estimates of antiretroviral coverage at mid-year were 3.0% in 2003, 4.9% in 2004, 10.0% in 2005, 19.7% in 2006, 28.3% in 2007 and 40.2% in 2008 [18]. We designated the initial

period of 2003–04 as the early-HAART era, the period 2005–06 as the intermediate-HAART era and 2007–08 was categorized as the established-HAART era.

### Invasive pneumococcal disease surveillance

An IPD episode was defined as identification of pneumococcus from a normally sterile site (e.g., cerebrospinal fluid [CSF], blood, pleural fluid or joint fluid). Repeat IPD episodes from the same individual more than 21 days apart were considered as new episodes. The standard culture methods at the hospital included blood and CSF cultured for pneumococcal growth with the BacT/Alert microbial detection system (Organon Teknica, Durham, NC). Autolysed blood culture specimens, macroscopically chocolate-coloured with or without pleomorphic Gram-positive cocci on microscopy, were tested with latex agglutination kit (Wellcogen Bacterial Antigen Kit, Remel Europe Ltd, Dartford, UK). As a quality control, blood cultures positive for pneumococcal antigen were confirmed by pneumococcal surface adhesin A (PsaA) PCR during 2004, and from January 2007 onwards at the surveillance reference laboratory. [20] A 99.7% (332/333) concordance was identified between the PCR and latex-agglutination test. [21] Peritoneal, pericardial and other fluid samples were processed according to standard culture procedures. [22]

IPD episodes were categorized as: i) “pneumococcal meningitis”: if pneumococci were cultured from CSF; if the latex antigen detection was positive on CSF; or when the CSF white cell count and/or biochemistry was suggestive of purulent meningitis with pneumococci isolated from blood; ii) “pneumococcal non-meningitis cases”: if pneumococci were detected from blood, pleural fluid or from sterile sites other than CSF in the absence of clinical or laboratory diagnosis of meningitis.

IPD episodes were recorded through a surveillance system under the auspices of the National Institute for Communicable Diseases (NICD), [23] and included prospective collection of clinical and demographic data. Audits comparing cases recorded by the NICD surveillance system against the hospital’s electronic laboratory database ensured record completeness of identification of all IPD episodes. Audits revealed that 99.0%, 93.6% and 92.4% of the IPD episodes in the early-, intermediate- and established-HAART eras, respectively, had been recorded by the NICD surveillance program. Cases not reported to the NICD, and identified through the hospital electronic database, were included in the final database following retrospective review of demographic characteristics.

### Determination of HIV infection status

HIV infection status was based on HIV testing done as standard-of-care by attending physicians. This was performed using HIV enzyme-linked immunosorbent assay (ELISA). For the primary analysis of IPD incidence, we inferred the HIV status in patients not tested for HIV by assuming that the prevalence of HIV infection was the same as for those who were tested, stratified by each of the three individual study periods. In a parallel analysis we calculated IPD incidences on individuals with confirmed HIV infection status. CD4+ cell counts taken within 3 months of the IPD episode were categorized into four groups: 0–100, 101–200, 201–350 and >350 cells per microliter.

### Estimation of HAART effectiveness

The screening method, based on the comparison of the proportion of cases with CD4+ cell count  $\leq 200$  cells per microliter on HAART and the proportion on HAART in the population, was used to estimate the effectiveness of HAART in the study population, using the formula: [24]

$$\text{HAART effectiveness} = 1 - \frac{\% \text{ of IPD cases in patients on HAART}}{1 - \% \text{ of IPD cases in patients on HAART}} \times \frac{1 - \% \text{ of population on HAART}}{\% \text{ of population on HAART}}$$

### Statistical analysis

Estimated incidence of IPD was calculated for the three HAART-eras stratified by age, gender and HIV status to yield stratum-specific incidence rates. Age groups were: 18–24 years-old, 25–44 years-old, 45–64 years-old and  $\geq 65$  years-old. Incidence risk ratios (IRR) were calculated by comparing group-specific incidence for the established- and early-HAART eras. Mortality was assessed as case-fatality ratio (CFR) (proportion of cases with known outcome who died during their hospitalization) and incidence of IPD-associated death.

Chi-square test and Fisher's exact test when necessary were used to compare the distribution of categorical variables. 95% confidence intervals (CIs) are reported and *p*-values  $< 0.05$  were

considered statistically significant. Continuous variables were compared using analysis of variance. Poisson regression was used to calculate CFR comparing patients with different CD4+ cell counts and 95% CIs adjusted for diagnosis. Demographic data and estimates of incidence and risk ratio were analysed using STATA version 11.0 (College Station, Texas, USA) and Epi Info 3.5.1 (CDC, Atlanta, Georgia, USA).

### Ethics

The surveillance study and site-specific analysis were approved by the Human Research Ethics Committee (Medical), from the University of the Witwatersrand, South Africa (protocol numbers M081117 and M090663, respectively). The overall surveillance approval is reviewed annually and includes getting written informed consent from all patients interviewed as part of the surveillance. On the cases detected through the regular retrospective audits no informed consent was required.

### Results

Overall, 2,567 IPD cases were identified from 2003 to 2008. This included 772 episodes during the early-HAART era, 900 in the intermediate-HAART era and 895 during the established-

**Table 1.** Baseline demographic and clinical features of adults with invasive pneumococcal disease during different periods of the HAART program.

|   | Early-HAART era | Intermediate-HAART era | Established -HAART era | p =     | 6-year period (2003–2008) |
|---|-----------------|------------------------|------------------------|---------|---------------------------|
| <b>Total isolates</b>   | <b>772</b>      | <b>900</b>             | <b>895</b>             |         | <b>2,567</b>              |
| <b>Age in years Mean (range)</b>                                |                 |                        |                        |         |                           |
| Overall   | 37.8 (18–85)    | 38.9 (18–86)           | 38.7 (18–85)           |         | 38.5 (18–86)              |
| Female  | 37.0 (18–81)    | 38.0 (18–86)           | 38.0 (18–85)           |         | 37.7 (18–86)              |
| Male  | 38.6 (19–85)    | 39.8 (18–85)           | 39.6 (18–82)           |         | 39.3 (18–85)              |
| HIV-uninfected  | 41.4 (18–85)    | 40.5 (18–83)           | 43.2 (20–79)           |         | 41.8 (18–85)              |
| HIV-infected  | 36.5 (18–79)    | 37.6 (18–76)           | 37.6 (18–77)           |         | 37.3 (18–79)              |
| HIV unknown   | 39.8 (18–85)    | 42.3 (18–86)           | 42.4 (18–85)           |         | 41.2 (18–86)              |
| <b>HIV information (%)</b>                                      |                 |                        |                        |         |                           |
| HIV-uninfected  | 48 (6.2)        | 57 (6.3)               | 66 (7.4)               | 0.33    | 171 (6.7)                 |
| HIV-infected  | 481 (62.3)      | 643 (71.4)             | 710 (79.3)             | <0.0001 | 1834 (71.4)               |
| HIV unknown   | 243 (31.5)      | 200 (22.2)             | 119 (13.3)             | <0.0001 | 562 (21.9)                |
| Extrapolated uninfected   | 70 (9.1)        | 73 (8.1)               | 76 (8.5)               | 0.70    | 219 (8.5)                 |
| Extrapolated infected   | 702 (90.9)      | 827 (91.9)             | 819 (91.5)             |         | 2348 (91.5)               |
| <b>Gender (%)</b>   |                 |                        |                        |         |                           |
| Female  | 387 (50.1)      | 491 (54.6)             | 510 (57.0)             | 0.005   | 1388 (54.1)               |
| Male  | 381 (49.4)      | 406 (45.1)             | 384 (42.9)             | 0.009   | 1171 (45.6)               |
| Unknown   | 4 (0.5)         | 3 (0.3)                | 1 (0.1)                | 0.14    | 8 (0.3)                   |
| <b>Range of <sup>1</sup>CD4+ cell count in HIV-infected (%)</b> |                 |                        |                        |         |                           |
| Patients with information                                       | 125             | 405                    | 493                    |         | 1023                      |
| 0–100   | 60 (48.0)       | 224 (55.3)             | 297 (60.2)             | 0.01    | 581 (56.8)                |
| 101–200   | 35 (28.0)       | 86 (21.2)              | 118 (23.9)             | 0.75    | 239 (23.4)                |
| 201–350   | 14 (11.2)       | 64 (15.8)              | 49 (9.9)               | 0.14    | 127 (12.4)                |
| >350  | 16 (12.8)       | 31 (7.7)               | 29 (5.9)               | 0.01    | 76 (7.4)                  |

Laboratory-confirmed cases of IPD admitted at Chris Hani Baragwanath Hospital, Soweto, South Africa, during 2003–2008. A total of 2,567 cases ranging from 18 to 86 years old were included in the study.

*p* values were obtained from a chi-square test for trend.

<sup>1</sup>CD4+ cell count obtained within 3 months before or after hospitalization.

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HAART era (Table 1). HIV status information was available for 2,005 cases (78.1%), including 529 (68.5%) in the early-HAART era, 700 (77.8%) in the intermediate-HAART era and 776 (86.7%) in the established-HAART era;  $p < 0.0001$ . Gender data was missing in 8 cases (0.3%), which were excluded from the gender-specific analysis.

The most common clinical syndromes associated with IPD were pneumococcal pneumonia (63.5%, pneumococci identified from pleural fluid or blood in individuals with a physician-based diagnosis of pneumonia), pneumococcal meningitis (21.2%), pneumococcal bacteraemia without focus (14.1%, pneumococci identified from blood without any localizing illness) and other IPD (1.3%, pneumococci identified from sterile sites other than blood or CSF in the absence of clinical or laboratory diagnosis of meningitis or pneumonia).

The proportion of IPD cases involving women increased from 50.1% in the early-HAART to 57.0% in the established-HAART era ( $p = 0.005$ ), which was especially apparent in the 25–44 year age-group (48.3% vs. 56.9%, respectively;  $p = 0.004$ ). The prevalence of HIV-infection was greater in women (1,033 [93.7%] of 1,102) than men (799 [88.8%] of 900;  $p = 0.0001$ ) among tested individuals. Females were younger ( $37.7 \pm 12.1$  years) than males ( $39.3 \pm 10.9$  years;  $p = 0.004$ ). HIV-infected patients were younger ( $37.3 \pm 9.8$  years) than HIV-uninfected patients ( $41.8 \pm 15.7$  years;  $p < 0.0001$ ), (Table 1). Patients with unknown HIV status compared to individuals with known HIV status were older (41.2 years vs. 37.7 years, respectively;  $p < 0.0001$ ) but were similar in gender status (51.3% vs. 55.0% female, respectively;  $p = 0.12$ ).

CD4+ cell counts obtained within 3 months of the IPD episode were available in 55.8% (1,023/1,834) of confirmed HIV-infected cases and more commonly available in the established- (69.4%) and intermediate- (63.0%) compared to the early-HAART era (26.0%;  $p < 0.0001$ ). HIV-infected adults with CD4+ cell count results were more likely to be categorized as having severe immunosuppression (CD4+ cell count  $\leq 100$  cells per microliter) in the established- (60.2%) and intermediate- (55.3%) than in the early-HAART era (48.0%;  $p = 0.01$ ) (Table 1). Conversely, a lower proportion of adults with IPD had CD4+ cell counts  $> 350$  cells per microliter in the established-HAART era (5.9%) compared to the early-HAART era (12.8%;  $p = 0.01$ ) (Table 1).

#### Incidence of culture-confirmed invasive pneumococcal disease

There was no change in the overall incidence (per 100,000 population) of IPD comparing the early- (52.3) to the established-HAART era (57.7;  $p = 0.05$ ) (Table 2). However, a 46.4% increase was observed in incidence of pneumococcal meningitis (9.4 vs. 13.8, respectively;  $p = 0.0004$ ), whereas the incidence of non-meningitis cases remained stable (42.9 vs. 43.9;  $p = 0.68$ ) comparing the early to the established-HAART era, respectively.

The overall incidence of IPD, pneumococcal meningitis and non-meningitis pneumococcal disease remained unchanged in HIV-uninfected adults across the three time periods (Table 2). Among HIV-infected adults, overall incidence of IPD did not change comparing the early- (207.5) to the established-HAART era (214.0;  $p = 0.55$ ). However, when stratifying by diagnosis there was a 47.9% increase in incidence of pneumococcal meningitis

**Table 2.** Incidence of overall invasive pneumococcal disease and by syndrome for the three study periods in adults aged  $\geq 18$  years.

|                              | Incidence of IPD, cases per 100,000 population |                          |                          | Change in incidence                   |          |
|------------------------------|--|--------------------------|--------------------------|---------------------------------------|----------|
|                              | Early-HAART; (N=)                              | Intermediate-HAART; (N=) | Established -HAART; (N=) | IRR (Established vs. Early); [95% CI] | $p = ^1$ |
| <b>All population</b>        |  |                          |                          |                                       |          |
| Overall IPD                  | 52.3 ; (772)                                   | 59.4 ; (900)             | 57.7 ; (895)             | 1.10 ; [1.00 ; 1.21]                  | 0.05     |
| Meningitis                   | 9.4 ; (139)                                    | 12.6 ; (191)             | 13.8 ; (214)             | 1.46 ; [1.18 ; 1.81]                  | 0.0004   |
| Non-Meningitis               | 42.9 ; (633)                                   | 46.8 ; (709)             | 43.9 ; (681)             | 1.02 ; [0.92 ; 1.14]                  | 0.68     |
| Male                         | 50.1 ; (381)                                   | 52.2 ; (406)             | 48.3 ; (384)             | 0.97 ; [0.84 ; 1.11]                  | 0.63     |
| Female                       | 54.2 ; (387)                                   | 66.7 ; (491)             | 67.3 ; (510)             | 1.24 ; [1.09 ; 1.42]                  | 0.001    |
| <b>HIV-infected adults</b>   |  |                          |                          |                                       |          |
| Overall IPD                  | 207.5 ; (702)                                  | 225.6 ; (827)            | 214.0 ; (819)            | 1.03 ; [0.93 ; 1.14]                  | 0.55     |
| Meningitis                   | 34.3 ; (116)                                   | 48.3 ; (177)             | 50.7 ; (194)             | 1.48 ; [1.17 ; 1.86]                  | 0.001    |
| Non-Meningitis               | 172.9 ; (585)                                  | 177.3 ; (650)            | 163.3 ; (625)            | 0.94 ; [0.84 ; 1.06]                  | 0.32     |
| Male                         | 200.6 ; (339)                                  | 197.7 ; (360)            | 180.6 ; (341)            | 0.90 ; [0.77 ; 1.05]                  | 0.17     |
| Female                       | 212.1 ; (359)                                  | 252.1 ; (465)            | 246.2 ; (477)            | 1.16 ; [1.01 ; 1.33]                  | 0.03     |
| <b>HIV-uninfected adults</b> |  |                          |                          |                                       |          |
| Overall IPD                  | 6.2 ; (70)                                     | 6.4 ; (73)               | 6.5 ; (76)               | 1.06 ; [0.76 ; 1.46]                  | 0.74     |
| Meningitis                   | 2.0 ; (23)                                     | 1.2 ; (14)               | 1.7 ; (20)               | 0.85 ; [0.46 ; 1.54]                  | 0.58     |
| Non-Meningitis               | 4.2 ; (48)                                     | 5.1 ; (59)               | 4.8 ; (56)               | 1.13 ; [0.77 ; 1.67]                  | 0.52     |
| Male                         | 7.1 ; (42)                                     | 7.7 ; (46)               | 7.1 ; (43)               | 1.0 ; [0.65 ; 1.53]                   | 0.99     |
| Female                       | 5.1 ; (28)                                     | 4.7 ; (47)               | 5.9 ; (33)               | 1.14 ; [0.69 ; 1.89]                  | 0.61     |

Incidence risk ratios and percentage of change in incidence of disease between the early- and established-HAART eras, assuming that the prevalence of HIV infection in untested cases is the same as in the tested cases.

<sup>1</sup>Chi-square test or Fischer-test.

IRR: incidence risk ratios established- vs. early-HAART era.

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**Table 3.** Incidence of invasive pneumococcal disease across the three periods, limited to adults with known HIV-infection status.

|                           | Incidence of IPD, cases per 100,000 population |                           |                          | Change in incidence                   |                  |
|---------------------------|--|---------------------------|--------------------------|---------------------------------------|------------------|
|                           | Early-HAART ; (N=)                             | Intermediate-HAART ; (N=) | Established -HAART; (N=) | IRR (Established vs. Early); [95% CI] | p = <sup>1</sup> |
| <b>Overall</b>            |  |                           |                          |                                       |                  |
| Tested HIV-infected       | 142.2 ; (481)                                  | 175.4 ; (643)             | 185.6 ; (710)            | 1.31 ; [1.16 ; 1.47]                  | <0.000           |
| Tested HIV-uninfected     | 4.2 ; (48)                                     | 5.0 ; (57)                | 5.6 ; (66)               | 1.34 ; [0.92 ; 1.94]                  | 0.12             |
| <b>18-24 years old</b>    |  |                           |                          |                                       |                  |
| Tested HIV-infected       | 66.3 ; (38)                                    | 76.2 ; (41)               | 97.3 ; (50)              | 1.47 ; [0.96 ; 2.23]                  | 0.07             |
| Tested HIV-uninfected     | 3.2 ; (8)                                      | 4.7 ; (11)                | 4.8 ; (11)               | 1.47 ; [0.59 ; 3.67]                  | 0.40             |
| <b>25-44 years old</b>    |  |                           |                          |                                       |                  |
| Tested HIV-infected       | 142.7 ; (346)                                  | 172.9 ; (459)             | 181.8 ; (504)            | 1.27 ; [1.11 ; 1.46]                  | 0.0005           |
| Tested HIV-uninfected     | 3.4 ; (18)                                     | 5.3 ; (28)                | 4.9 ; (26)               | 1.44 ; [0.79 ; 2.62]                  | 0.24             |
| <b>45-64 years old</b>    |  |                           |                          |                                       |                  |
| Tested HIV-infected       | 247.9 ; (95)                                   | 284.7 ; (133)             | 279.3 ; (148)            | 1.13 ; [0.87 ; 1.46]                  | 0.36             |
| Tested HIV-uninfected     | 6.5 ; (18)                                     | 3.8 ; (11)                | 7.0 ; (21)               | 1.07 ; [0.57 ; 2.00]                  | 0.84             |
| <b>&gt;= 65 years old</b> |  |                           |                          |                                       |                  |
| Tested HIV-infected       | 799.3 ; (2)                                    | 1,927.6 ; (10)            | 834.9 ; (8)              | 1.05 ; [0.22 ; 4.88]                  | 1.00             |
| Tested HIV-uninfected     | 4.5 ; (4)                                      | 7.2 ; (7)                 | 7.4 ; (8)                | 1.64 ; [0.49 ; 5.45]                  | 0.57             |

Incidence risk ratios and percentage of change in incidence of disease between the early- and established-HAART eras.

<sup>1</sup>Chi-square test or Fischer-test.

IRR: incidence risk ratios established- vs. early-HAART era.

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between the early- (34.3) compared to the established-HAART era (50.7;  $p=0.001$ ), whereas the incidence of non-meningitis disease remained the same between the early- and established-HAART era (172.9 vs. 163.3, respectively;  $p=0.32$ ), (Table 2). The risk of IPD was 33.7-fold (95% CI: 26.4; 43.1) greater in HIV-infected than in HIV-uninfected adults during the early-HAART era and this increased risk persisted (IRR: 32.9; 95% CI: 26.0; 41.7) in the established-HAART era.

Stratification by gender revealed a 16.1% increase in incidence of IPD in HIV-infected females from the early-HAART era (212.1) compared to the established-HAART era (246.2;  $p=0.03$ ), whilst the incidence of IPD remained stable in HIV-infected males (Table 2). The increase in incidence of IPD in HIV-infected females was mainly due to an 18.7% increase in the 25–44 years age-group (204.2 vs. 242.4;  $p=0.04$ ) (Table S1). In addition, HIV-infected females had 1.4 (95% CI: 1.2; 1.6) greater risk of IPD than HIV-infected males in the established-HAART era;  $p<0.0001$ . Although a decrease in incidence of IPD was observed in HIV-infected individuals older than 65 years comparing the early (3596.8) to established HAART-era (1565;  $p=0.04$ ), these individuals accounted for 1.3% and 1.8% of all IPD cases in the respective periods. A detailed analysis of the incidence of IPD stratified by age-group, HIV status and gender is reported in online Table S1.

The analysis considering only individuals tested for HIV also showed an overall increase in incidence (per 100,000) of IPD from the early- (142.2) to established-HAART era (185.6;  $p<0.0001$ ) in HIV-infected adults (Table 3). The incidence of IPD, however, remained unchanged from the early- (4.2) compared to established-HAART era (5.6) in HIV-uninfected adults based on confirmed HIV-seronegativity status of cases. Significant increase in IPD, based on confirmed HIV-seropositivity of cases, from the early- to established-HAART era was observed in adults in the 25–44 age-group strata (142.7 vs. 181.8,  $p=0.0005$ ) and a similar

trend was observed in the 18–24 years age-group strata (66.3 vs. 97.3;  $p=0.07$ ) (Table 3). Although there were similar trends in increase in incidence of IPD among the HIV-uninfected group, based on documented HIV-seronegativity of cases, these were not significant and the incidence of IPD remained less than 8 cases per 100,000 overall and in all age-groups across all study periods (Table 3).

From the early- to the established-HAART era, the percentage of cases detected on blood samples by latex agglutination/PCR increased from 27.2% to 43.2% of the total cases, respectively;  $p<0.0001$ . Since this change in laboratory procedures could have influenced our results we recalculated the incidence of pneumococcal meningitis in adults including only episodes with culture of pneumococcus from CSF (Table 4). Incidence of CSF-culture-confirmed pneumococcal meningitis increased by 42.5% from 33.7 in the early-HAART to 48.0 in the established-HAART era in HIV-infected females ( $p=0.03$ ).

#### Disease outcome

IPD clinical outcome data was unknown or missing in 366 cases (14.3%). The in-hospital case fatality ratio (CFR) did not change during the study period (30.2% in the early-HAART era, 34.1% in the intermediate-HAART era and 31.6% in the established-HAART era;  $p=0.66$ ). The overall CFR was similar in HIV-infected (29.2%) and HIV-uninfected persons (29.4%;  $p=0.95$ ) (Table 5). Among HIV-infected patients, those with meningitis had a higher CFR (55.2%) compared to those with a non-meningitis diagnosis (22.4%,  $p<0.0001$ ) (Table 5). Similarly HIV-uninfected individuals with meningitis had a higher CFR (43.9%) compared with non-meningitis patients (24.1%;  $p=0.02$ ). A higher CFR was, however, observed in adults with non-meningitis pneumococcal disease in whom HIV infection status was not established (39.3%) compared to those with known HIV infection status (22.5%;  $p<0.0001$ ).

**Table 4.** Incidence of CSF-culture-confirmed pneumococcal meningitis for the three study periods in adults aged  $\geq 18$  years.

|                              | Incidence of IPD, cases per 100,000 population |                          |                          | Change in incidence                   |                  |
|------------------------------|--|--------------------------|--------------------------|---------------------------------------|------------------|
|                              | Early-HAART; (N=)                              | Intermediate-HAART; (N=) | Established -HAART; (N=) | IRR (Established vs. Early); [95% CI] | p = <sup>1</sup> |
| <b>All population</b>        |  |                          |                          |                                       |                  |
| Overall                      | 8.6 ; (127)                                    | 10.9 ; (165)             | 10.1 ; (156)             | 0.86 ; [0.68 ; 1.08]                  | 0.19             |
| Male                         | 8.0 ; (61)                                     | 9.1 ; (71)               | 7.4 ; (59)               | 1.08 ; [0.75 ; 1.54]                  | 0.68             |
| Female                       | 9.0 ; (64)                                     | 12.8 ; (94)              | 12.8 ; (97)              | 0.70 ; [0.51 ; 0.96]                  | 0.03             |
| <b>HIV-infected adults</b>   |  |                          |                          |                                       |                  |
| All population               | 30.7 ; (104)                                   | 41.7 ; (153)             | 36.9 ; (141)             | 0.83 ; [0.65 ; 1.07]                  | 0.16             |
| Male                         | 26.6 ; (45)                                    | 35.7 ; (65)              | 25.4 ; (48)              | 1.05 ; [0.70 ; 1.57]                  | 0.82             |
| Female                       | 33.7 ; (57)                                    | 47.7 ; (88)              | 48.0 ; (93)              | 0.70 ; [0.50 ; 0.98]                  | 0.03             |
| <b>HIV-uninfected adults</b> |  |                          |                          |                                       |                  |
| All population               | 2.0 ; (23)                                     | 1.0 ; (12)               | 1.3 ; (15)               | 1.58 ; [0.82 ; 3.02]                  | 0.17             |
| Male                         | 2.7 ; (16)                                     | 1.0 ; (6)                | 1.8 ; (11)               | 1.49 ; [0.69 ; 3.21]                  | 0.31             |
| Female                       | 1.3 ; (7)                                      | 1.1 ; (6)                | 0.7 ; (4)                | 1.81 ; [0.53 ; 6.18]                  | 0.34             |

Incidence risk ratios and percentage of change in incidence of disease between the early- and established-HAART eras, assuming that the prevalence of HIV infection in untested cases is the same as in the tested cases.

<sup>1</sup>Chi-square test or Fischer-test.

IRR: incidence risk ratios established- vs. early-HAART era.

95% CI: 95% confidence interval.

HAART: highly active anti-retroviral treatment.

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**Table 5.** Case fatality ratios and incidence of mortality during the three study periods in IPD patients by HIV status and diagnosis.

|   | Early-HAART era   | Intermediate-HAART era | Established -HAART era | p =               | 6-year period (2003-2008) |
|---|-------------------|------------------------|------------------------|-------------------|---------------------------|
| <b>Cases with known outcome (%)</b>         | <b>616 (79.8)</b> | <b>772 (85.8)</b>      | <b>813 (90.8)</b>      | <b>&lt;0.0001</b> | <b>2,201 (85.7)</b>       |
| <b>Died during hospitalization (%)</b>      |                   |                        |                        |                   |                           |
| Overall                                     | 186 (30.2)        | 263 (34.1)             | 257 (31.6)             | 0.66              | 706 (32.1)                |
| Tested HIV-infected                         | 109 (26.3)        | 176 (30.6)             | 199 (29.8)             | 0.28              | 484 (29.2)                |
| Tested HIV-uninfected                       | 9 (22.0)          | 19 (40.4)              | 17 (26.2)              | 0.84              | 45 (29.4)                 |
| HIV unknown                                 | 68 (42.2)         | 68 (45.6)              | 41 (51.3)              | 0.19              | 177 (45.4)                |
| Meningitis HIV-infected                     | 35 (56.5)         | 70 (58.3)              | 85 (52.5)              | 0.45              | 190 (55.2)                |
| Meningitis HIV-uninfected                   | 5 (38.5)          | 6 (60.0)               | 7 (38.9)               | 0.94              | 18 (43.9)                 |
| Meningitis HIV unknown                      | 25 (65.8)         | 25 (55.6)              | 15 (68.2)              | 0.98              | 65 (61.9)                 |
| Non-meningitis HIV-infected                 | 74 (21.0)         | 106 (23.2)             | 114 (22.5)             | 0.65              | 294 (22.4)                |
| Non-meningitis HIV-uninfected               | 4 (14.3)          | 13 (35.1)              | 10 (21.3)              | 0.70              | 27 (24.1)                 |
| Non-meningitis HIV unknown                  | 43 (35.0)         | 43 (41.3)              | 26 (44.8)              | 0.18              | 112 (39.3)                |
| <b>Incidence of mortality (per 100,000)</b> |                   |                        |                        |                   |                           |
| Overall                                     | 12.6              | 17.4                   | 16.6                   | 0.006             |                           |
| Tested HIV-infected                         | 32.2              | 48.0                   | 52.0                   | 0.0001            |                           |
| Tested HIV-uninfected                       | 0.8               | 1.7                    | 1.5                    | 0.17              |                           |
| Meningitis HIV-infected                     | 10.3              | 19.1                   | 22.2                   | 0.0001            |                           |
| Meningitis HIV-uninfected                   | 0.44              | 0.52                   | 0.60                   | 0.60              |                           |
| Non-meningitis HIV-infected                 | 21.9              | 28.9                   | 29.8                   | 0.04              |                           |
| Non-meningitis HIV-uninfected               | 0.35              | 1.1                    | 0.86                   | 0.18              |                           |

p values were obtained from a chi-square test for trend.

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HIV-infected adults with CD4+ cell counts  $\leq 100$  cells per microliter had a higher CFR compared to those with CD4+ cell counts  $>100$  cells per microliter for meningitis cases (57.8% [63/109] vs. 38.8% [31/80], respectively;  $p=0.01$ ) as well as for non-meningitis cases (29.4% [124/422] vs. 9.8% [32/328],  $p<0.0001$ ). Overall, a higher CFR was observed in HIV-infected adults with CD4+ cell counts  $\leq 100$  cells per microliter (35.2%; [186/529]) than those with CD4+ cell counts  $>100$  cells per microliter (15.5%; [63/406]) (adjusted IRR for disease syndrome 2.3 [95% CI: 1.7; 3.0];  $p<0.0001$ ). No difference in CFR was observed between females and males after adjusting for CD4+ cell counts and disease syndrome.

The overall incidence of IPD-associated mortality (per 100,000) increased, based on documented HIV infection status of cases, in HIV-infected adults from the early- (32.2) compared to established-HAART (52.0;  $p=0.0001$ ) period. Similarly, increases in mortality incidence were observed for meningitis (10.3 vs. 22.2;  $p=0.0001$ ) and non-meningitis (21.9 vs. 29.8;  $p=0.04$ ) in confirmed HIV-infected adults. The mortality rates did not change significantly in HIV-uninfected individuals, based on confirmed HIV-negative cases, (Table 5).

### HAART effectiveness

During 2008, 40% of AIDS patients (defined by CD4+ cell count less than 200 cells per microliter or WHO stage 4) were estimated to be on HAART in South Africa. [14] During 2008, 230 IPD HIV-infected patients had CD4+ cell counts  $\leq 200$  cell per microliter being eligible for HAART according to the national guidelines. [19] HAART information was available on 192 of these IPD cases from 2008 (175 cases not on HAART and 17 cases on HAART). Consequently the frequency of IPD cases that occurred in the HAART treated group was 8.9%. Using the screening method [24] and based on estimates of HAART coverage and our study findings, HAART was 85.5% effective in reducing IPD in HIV-infected adults.

### Discussion

Unlike reports from developed countries [6,7] and a recent study from Malawi [9] our study failed to show any impact of the antiretroviral treatment program on the burden of IPD in HIV-infected adults. This result was largely attributed to an increase in IPD incidence observed in HIV-infected women, especially women aged 25–44 years. In addition to the majority (71.4%) of IPD cases occurring in adults confirmed to be HIV-infected, the risk of IPD remained consistently 33- to 34-fold greater in HIV-infected compared to -uninfected adults over the study period. The baseline estimate of IPD, i.e. in the early-HAART era, observed in our study was similar to previous reports from the same study population in 1997 when HAART was not being used. The incidence of IPD in adults in this population in the latter study was 45 cases per 100,000 in the 25–44 years old, 42 cases per 100,000 in the 45–64 years old and 50 cases per 100,000 in adults older than 65 years. [16]

The high burden of IPD in the study population, attributed to the HIV epidemic, is further emphasized by the increase in overall and HIV-confirmed incidence of IPD-associated mortality rates in adults. Case fatality ratios of IPD among HIV-infected patients vary widely according to the population studied. Several studies that compare mortality between HIV-infected (9%–21%) and HIV-uninfected (3%–15%) individuals observed no significant differences in mortality in the two populations [8,15,16] and even the degree of CD4+ cell depletion did not influence mortality rates in IPD. [25] HIV-infected adults in our study with CD4+ cell counts  $\leq 100$  cells per microliter, however, had a higher in hospital

CFR (35%), after adjusting for disease syndrome, compared to those with CD4+ cell counts  $>100$  cells per microliter.

A similar analysis on the trends of IPD incidence in South African children and teenagers revealed that up-scaling of the HAART program in HIV-infected children was associated with a 50.8% reduction in IPD hospitalization and 65.2% reduction in IPD-associated mortality rates in the same population. [26] Reductions were evident for both pneumococcal meningitis and pneumococcal non-meningitis disease. [26] This suggests that the HAART program among children has been more successful in its implementation and management in this community compared to its scale and coverage in adults.

Using the incidence of IPD as a proxy measure of the effectiveness of the HAART program in reducing opportunistic-infections at a population level in HIV-infected adults, our study indicates an ongoing high prevalence of immunosuppression in this community. Nevertheless we used the screening method to estimate the likely effectiveness of HAART based on the prevalence of HAART coverage in HIV-infected cases. [24] This method relies on external standardization and depends on the accuracy of the external estimates used. We used a conservative estimate of HAART coverage derived from a study by Adam et al., where combined CD4+ cell count and clinical criteria were used to define HAART eligibility. [18] Other sources, specifically the ASSA2003 model, [14] commonly used in extrapolating HAART targets in South Africa assume patients in need in terms of WHO clinical staging only, produced higher estimates of coverage. We estimated that even though adults on HAART are at reduced risk of IPD, an effect was not detected at the population level. These seemingly contradictory findings might be the result of number of factors, including: i) over-estimates of HAART coverage in the population; ii) a maturation in the HIV epidemic, including a larger population with progression to severe immunosuppression; iii) possible inadequacies in the HAART program with regard to patient management and compliance; and iv) delay in accessing of HAART treatment by severely immunocompromised HIV-infected individuals.

A limitation of applying the screening method in evaluating the effectiveness of HAART in our study, however, include that acute infections such as the IPD may have caused a transient reduction in CD4+ counts. Hence, the proportion of IPD cases who purportedly qualified for HAART may have been over-estimated.

A small cohort study in HIV-infected adults, in a poor inner city population in Baltimore (USA) between 1990 to 2003 also did not observe any change in incidence of IPD despite widespread implementation of HAART. [27] In addition, another USA study involving nation-wide hospitalization trends of IPD among HIV-infected subjects, whilst observing a marked decrease in the rate of IPD hospitalizations from 1994 to 2005, after adjusting for age, sex and payer the decline was only apparent after introduction of childhood PCV vaccination and more likely to have been attributable to indirect protection conferred by the childhood PCV immunization program. [28]

Although an analysis from Malawi reported that increase use of antiretroviral was associated with a decline in IPD, the results were confounded by changes in the method of surveillance of IPD prior to and after the establishment of the HAART program. [9] In addition the lack of population denominators and HIV specific data, limited the scope of the analysis in attributing the reduction in IPD cases to the HAART program. [9]

Our study has potential limitations, including those which are inherent to any longitudinal observational/time-series analysis. These include the possibility of changes in practice in both clinical practice, as well as surveillance systems over time. Improvements



in surveillance and clinical care over the surveillance periods in our study included an increase in the proportion of cases in whom outcome, HIV-infection and CD4+ cell counts data were available. In the early-HAART era HIV was possible under-diagnosed and consequently may have falsely lead to over-estimate of IPD in HIV-uninfected and under-estimate in HIV-infected subjects during that period.

Although changes in clinical practices by attending physicians (i.e. likelihood of performing blood cultures) may had also influence our observations, a measure of IPD which is least subject to changes in patient-management is microbiologically confirmed meningitis. The increase in incidence in pneumococcal-meningitis and especially in meningitis culture confirmed on CSF from the early- compared to the established-HAART era in HIV-infected adults, whilst remaining unchanged in HIV-uninfected adults, corroborates our findings of an overall increase in IPD in HIV-infected adults as being plausible.

The ongoing high burden of IPD in HIV-infected adults in our study population indicates an urgent need for additional approaches to prevent pneumococcal disease. Although expanding and optimizing HAART therapy is likely to be most effective in reducing the risk of opportunistic infections in these adults, other strategies are needed. One such strategy, which is recommended by WHO and included in the South African HIV/AIDS treatment guidelines, is cotrimoxazole prophylaxis. [29] Cotrimoxazole has been shown to reduce the burden of bacterial infections in African HIV-infected adults, albeit not specifically confirmed pneumococcal disease. [30,31] Of concern, however, has been the increase in prevalence of antibiotic non-susceptible pneumococcal strains involving nasopharyngeal colonization and IPD in HIV-infected subjects on prophylaxis. [32,33]

A different strategy of decreasing the burden of IPD in HIV-infected adults includes the possibility of vaccination against pneumococcal disease. The use of PPV23 in African HIV-infected adults is controversial, based on a Ugandan study which showed an increase in pneumonia in the short-term period following PPV23 vaccination of HIV-infected adults not on HAART [34] albeit subsequently showing a 16% reduction in all-cause mortality. [35] The lack of PPV23 efficacy against pneumonia observed in the study by French et al. probably relates to their cohort of PPV23 vaccinees mainly having high viral loads (>100,000 copies per millilitre), [34] which has been established as an important factor for lack of PPV23 effectiveness even in the USA. [36] Nevertheless, PPV23 vaccination is not routinely undertaken in South African HIV-infected adults or

recommended by the WHO for African HIV-infected adults. More recently a 7-valent PCV (PCV7) was established to be safe and efficacious in preventing vaccine-serotype specific IPD in African HIV-infected adults with previously documented history of IPD, many (86%) of whom were not on HAART. [37]

Another possibility for reducing the burden of IPD, of at least select serotypes included in PCVs formulations, in HIV-infected and -uninfected adults is through indirect protection from childhood PCV vaccination. This may be achieved by immunization of large proportions of young children in the community with PCV. Experience from the USA indicated that widespread immunization of young children with PCV7 was associated with a 69% reduction in vaccine-serotype IPD in select adult age groups, including a 91% reduction in HIV-infected adults. [38,39] PCV7 was introduced in the South African public childhood immunization program in April 2009 and the effect thereof on protection of HIV-infected adults is being studied.

There have as yet been no studies clearly demonstrating a reduction in the incidence any opportunistic infections among HIV-infected adults in the HAART era in SA. As HAART coverage increases ongoing surveillance will clarify the situation. However adjunctive policies appear to be warranted in the prevention of IPD.

## Supporting Information

**Table S1 Incidence of overall invasive pneumococcal disease across study periods in adults stratified by age, gender and HIV infection status.** (DOCX)

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## Author Contributions

Analyzed the data: MCN LK. Contributed reagents/materials/analysis tools: ASK LdG AvG. Wrote the paper: MN SAM. Analysis design: MCN SAM AvG KPK LK CC. Acquired and managed the data: ASK LdG AvG.

## References

- Bliss SJ, O'Brien KL, Janoff EN, Cotton MF, Musoke P, et al. (2008) The evidence for using conjugate vaccines to protect HIV-infected children against pneumococcal disease. *Lancet Infect Dis* 8: 67–80.
- Jones N, Huebner R, Khoosal M, Crewe-Brown H, Klugman K (1998) The impact of HIV on *Streptococcus pneumoniae* bacteraemia in a South African population. *Aids* 12: 2177–2184.
- McEllistrem MC, Mendelsohn AB, Pass MA, Elliott JA, Whitney CG, et al. (2002) Recurrent invasive pneumococcal disease in individuals with human immunodeficiency virus infection. *J Infect Dis* 185: 1364–1368.
- Redd SC, Rutherford GW, 3rd, Sande MA, Lifson AR, Hadley WK, et al. (1990) The role of human immunodeficiency virus infection in pneumococcal bacteremia in San Francisco residents. *J Infect Dis* 162: 1012–1017.
- Palella FJ, Jr., Delaney KM, Moorman AC, Loveless MO, Fuhrer J, et al. (1998) Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med* 338: 853–860.
- Grau I, Pallares R, Tubau F, Schulze MH, Llopis F, et al. (2005) Epidemiologic changes in bacteremic pneumococcal disease in patients with human immunodeficiency virus in the era of highly active antiretroviral therapy. *Arch Intern Med* 165: 1533–1540.
- Heffernan RT, Barrett NL, Gallagher KM, Hadler JL, Harrison LH, et al. (2005) Declining incidence of invasive *Streptococcus pneumoniae* infections among persons with AIDS in an era of highly active antiretroviral therapy, 1995–2000. *J Infect Dis* 191: 2038–2045.
- Nuorti JP, Butler JC, Gelling L, Kool JL, Reingold AL, et al. (2000) Epidemiologic relation between HIV and invasive pneumococcal disease in San Francisco County, California. *Ann Intern Med* 132: 182–190.
- Everett DB, Mukaka M, Denis B, Gordon SB, Carrol ED, et al. (2011) Ten Years of Surveillance for Invasive *Streptococcus pneumoniae* during the Era of Antiretroviral Scale-Up and Cotrimoxazole Prophylaxis in Malawi. *PLoS One* 6: e17765.
- Mayanja BN, Todd J, Hughes P, Van der Paal L, Mugisha JO, et al. (2010) Septicaemia in a population-based HIV clinical cohort in rural Uganda, 1996–2007: incidence, aetiology, antimicrobial drug resistance and impact of antiretroviral therapy. *Trop Med Int Health* 15: 697–705.
- Operational Plan for Comprehensive HIV, AIDS Care, Management and Treatment for SA. Pretoria: National department of Health (2003) South African Government website. Available: <http://www.info.gov.za/issues/hiv/careplan.htm>. Accessed 2011 Feb.
- Shisana O, Simbayi LC, Rehle T, Zungu NP, Zuma K, et al. (2010) South African National HIV Prevalence, Incidence, Behaviour and Communication Survey, 2008: The health of our children. Cape Town: HSRC Press website. Available: <http://www.hsrepress.ac.za>. Accessed 2011 Feb.
- Shisana O, Rehle T, Simbayi LC, Parker W, Zuma K, et al. (2005) South African National HIV Prevalence, HIV Incidence, Behaviour and Communi-

- cation Survey, 2005. Cape Town: HSRC Press website. Available: <http://www.hsrbpress.ac.za>. Accessed 2011 Feb.
14. ASSA2003 Full and Provincial AIDS and Demographic Models. Actuarial Society of South Africa. Relevant documentation about the model is available at the Actuarial Society of South Africa website: <http://www.assa.org.za>. Accessed 2011 Feb.
  15. Feldman C, Glatthaar M, Morar R, Mahomed AG, Kaka S, et al. (1999) Bacteremic pneumococcal pneumonia in HIV-seropositive and HIV-seronegative adults. *Chest* 116: 107–114.
  16. Karstaedt AS, Khoosal M, Crewe-Brown HH (2001) Pneumococcal bacteremia in adults in Soweto, South Africa, during the course of a decade. *Clin Infect Dis* 33: 610–614.
  17. STATSSA, Mid-year population estimates 2003–2008. STATSSA website. Available: <http://www.statssa.gov.za>. Accessed 2011 Feb.
  18. Adam MA, Johnson LF (2009) Estimation of adult antiretroviral treatment coverage in South Africa. *S Afr Med J* 99: 661–667.
  19. National Department of Health South Africa website. Available: <http://www.doh.gov.za/docs/factsheets/guidelines/artguidelines04/intro.pdf>. Accessed 2011 Feb.
  20. Morrison KE, Lake D, Crook J, Carlone GM, Ades E, et al. (2000) Confirmation of *psaA* in all 90 serotypes of *Streptococcus pneumoniae* by PCR and potential of this assay for identification and diagnosis. *J Clin Microbiol* 38: 434–437.
  21. Radstrom P, Backman A, Qjan N, Kraghsbjerg P, Pahlson C, et al. (1994) Detection of bacterial DNA in cerebrospinal fluid by an assay for simultaneous detection of *Neisseria meningitidis*, *Haemophilus influenzae*, and streptococci using a seminested PCR strategy. *J Clin Microbiol* 32: 2738–2744.
  22. Winn W, Allen S, Janda W, Koneman E, Procop G, et al. (2006) Chapter 2: Introduction to Microbiology: Part II: Guidelines for the collection, transport, processing, analysis and reporting of cultures from specific specimen sources. editors Koneman's color atlas and textbook of diagnostic microbiology 6 ed Baltimore, MD, USA: Lippincott Williams & Wilkins, pp 68–105.
  23. Huebner RE, Klugman KP, Matai U, Eggers R, Hussey G (1999) Laboratory surveillance for *Haemophilus influenzae* type B meningococcal, and pneumococcal disease. Haemophilus Surveillance Working Group. *S Afr Med J* 89: 924–925.
  24. Farrington CP (1993) Estimation of vaccine effectiveness using the screening method. *Int J Epidemiol* 22: 742–746.
  25. Jordano Q, Falco V, Almirante B, Planes AM, del Valle O, et al. (2004) Invasive pneumococcal disease in patients infected with HIV: still a threat in the era of highly active antiretroviral therapy. *Clin Infect Dis* 38: 1623–1628.
  26. Nunes MC, von Gottberg A, de Gouveia L, Cohen C, Moore DP, et al. (2011) The impact of antiretroviral treatment on the burden of invasive pneumococcal disease in South African children: a time series analysis. *AIDS* 25: 453–462.
  27. Barry PM, Zetola N, Keruly JC, Moore RD, Gebo KA, et al. (2006) Invasive pneumococcal disease in a cohort of HIV-infected adults: incidence and risk factors, 1990–2003. *AIDS* 20: 437–444.
  28. Kourtis AP, Ellington S, Bansil P, Jamison DJ, Posner SF (2010) Hospitalizations for invasive pneumococcal disease among HIV-1-infected adolescents and adults in the United States in the era of highly active antiretroviral therapy and the conjugate pneumococcal vaccine. *J Acquir Immune Defic Syndr* 55: 128–131.
  29. World Health Organization/UNAIDS. (2001) Provisional WHO/UNAIDS recommendations on the use of cotrimoxazole prophylaxis in adults and children living with HIV/AIDS in Africa. *Afr Health Sci* 1: 30–31.
  30. Anglaret X, Chene G, Attia A, Toure S, Lafont S, et al. (1999) Early chemoprophylaxis with trimethoprim-sulphamethoxazole for HIV-1-infected adults in Abidjan, Cote d'Ivoire: a randomised trial. Cotrimo-CI Study Group. *Lancet* 353: 1463–1468.
  31. Wiktor SZ, Sassin-Morokro M, Grant AD, Abouya L, Karon JM, et al. (1999) Efficacy of trimethoprim-sulphamethoxazole prophylaxis to decrease morbidity and mortality in HIV-1-infected patients with tuberculosis in Abidjan, Cote d'Ivoire: a randomised controlled trial. *Lancet* 353: 1469–1475.
  32. Gill CJ, Mwanakasale V, Fox MP, Chilengi R, Tembo M, et al. (2008) Effect of presumptive co-trimoxazole prophylaxis on pneumococcal colonization rates, seroepidemiology and antibiotic resistance in Zambian infants: a longitudinal cohort study. *Bull World Health Organ* 86: 929–938.
  33. Pemba L, Charalambous S, von Gottberg A, Magalla B, Moloi V, et al. (2008) Impact of cotrimoxazole on non-susceptibility to antibiotics in *Streptococcus pneumoniae* carriage isolates among HIV-infected mineworkers in South Africa. *J Infect* 56: 171–178.
  34. French N, Nakiyingi J, Carpenter LM, Lugada E, Watera C, et al. (2000) 23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial. *Lancet* 355: 2106–2111.
  35. Watera C, Nakiyingi J, Miro G, Muwonge R, Whitworth JA, et al. (2004) 23-Valent pneumococcal polysaccharide vaccine in HIV-infected Ugandan adults: 6-year follow-up of a clinical trial cohort. *AIDS* 18: 1210–1213.
  36. Teshale EH, Hanson D, Flannery B, Phares C, Wolfe M, et al. (2008) Effectiveness of 23-valent polysaccharide pneumococcal vaccine on pneumonia in HIV-infected adults in the United States, 1998–2003. *Vaccine* 26: 5830–5834.
  37. French N, Gordon SB, Mwalukomo T, White SA, Mwafulirwa G, et al. (2010) A trial of a 7-valent pneumococcal conjugate vaccine in HIV-infected adults. *N Engl J Med* 362: 812–822.
  38. Cohen AL, Harrison LH, Farley MM, Reingold AL, Hadler J, et al. (2010) Prevention of invasive pneumococcal disease among HIV-infected adults in the era of childhood pneumococcal immunization. *AIDS* 24: 2253–2262.
  39. Centers for Disease Control and Prevention. (2005) Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease—United States, 1998–2003. *MMWR Morb Mortal Wkly Rep* 54: 893–897.





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## Immunogenicity of 13-valent pneumococcal conjugate vaccine among children with underlying medical conditions



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### ABSTRACT

**Background:** *Streptococcus pneumoniae* is a leading cause of vaccine-preventable disease in children under 5 years. Immunocompromised children and those with underlying diseases are at increased risk of severe complications from vaccine-preventable infections. We studied the humoral immune response to the 13-valent pneumococcal conjugate vaccine (PCV13) in children with HIV-infection, kidney or lung disease and compared this to the response in healthy control children.

**Methods:** Children aged 12–71 months with underlying conditions including HIV-infection and those with kidney and lung diseases (at-risk children), and a healthy control group were vaccinated with PCV13. The at-risk children received two doses of PCV13 and the controls received one dose. Serotype-specific antibodies for all PCV13 serotypes were measured by a luminex-based enzyme immunoassay at baseline and post-vaccination.

**Results:** After the first PCV13 dose, the fold-increase in serotype-specific antibody geometric mean concentrations (GMCs) from baseline and the percentage of participants with  $\geq 4$ -fold-increase in antibody concentrations was similar between the control and at-risk children. GMCs were, however, lower for three of the 13 serotypes in HIV-infected children, higher for serotype 6B in children with kidney disease and higher for serotypes 6B and 14 in children with lung disease. After second vaccine dose HIV-infected children had an increase in GMCs from post-first dose for nine serotypes but the percentage of participants with  $\geq 4$ -fold-increase from baseline was similar post-second dose compared to post-first dose except for serotypes 6A and 19F. In children with kidney or lung diseases the immune responses after second vaccine dose were similar to post-first dose. Attenuated responses were observed for serotypes 3 and 19A in all study-groups, which was especially pronounced in the at-risk groups.

**Conclusion:** All study-groups mounted an immune response to PCV13, with the at-risk groups having responses that were mostly similar to the control children.

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### 1. Introduction

*Streptococcus pneumoniae* is a leading cause of vaccine-preventable disease in children under 5 years of age [1]. 7-valent pneumococcal conjugate vaccine (PCV7) and an investigational 9-valent PCV (PCV9) demonstrated efficacy and effectiveness against invasive pneumococcal disease (IPD), clinical pneumonia and

death in children [1–8]; however, substantial IPD cases still occurred due to non-PCV7 serotypes, after global PCV7 rollout [9–12]. This led to the development of a 13-valent PCV (PCV13), containing the PCV7 serotypes (4, 6B, 9V, 14, 18C, 19F and 23F) and six additional serotypes (1, 3, 5, 6A, 7F and 19A) [13]. In 2010, 82% of IPD cases in South African children <5 years old were caused by the serotypes included in PCV13 [14,15].

In South Africa, PCV7 was first introduced in April 2009 as a 2 + 1 schedule for infants at 6 weeks, 14 weeks, and 9 months of age. PCV7 was replaced with PCV13 in April 2011, with gradual up-scaling of coverage by the end of 2011 [16]. PCV7 was introduced with no catch-up strategy implemented; however, a catch-up

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program was recommended following transition to PCV13, which included all children between 18 and 36 months of age; and children 36 and 71 months of age with underlying medical conditions, including immunocompromising conditions and chronic diseases [17].

Although PCVs have been established to be safe, immunogenic and efficacious in select groups of children with underlying medical conditions, including those with HIV/AIDS and malnutrition [14,18], further research is warranted to determine the optimal dosing schedule in these populations. The aim of this study was to determine the immunogenicity of one and two doses of PCV13 in pneumococcal vaccine-naïve children aged 12–71 months with underlying medical conditions.

## 2. Materials and methods

An open-label study was conducted at Chris Hani Baragwanath Academic Hospital (CHBAH) in Soweto, South Africa, where children with chronic medical or immunocompromising conditions are followed-up at specialist clinics. With the transition from PCV7 to PCV13 a catch-up plan was developed in early 2012 and all parents/caregivers were encouraged to take their children to health facilities across South Africa to be vaccinated. Enrolment into the study targeted “at-risk” children aged 12–71 months old presenting at the HIV, Renal and Pulmonology clinics, and healthy control children in the same age group identified through the PCV13 catch-up program. Exclusion criteria for study enrolment included prior receipt of any pneumococcal vaccination. Enrolment took place from 30 July 2012 to 9 May 2013.

### 2.1. Study procedures

At-risk children were administered two doses of PCV13, two months apart, while control children received only one PCV13 dose at enrolment. Venous blood samples were collected on four occasions from the at-risk children and on three occasions from controls: just prior to each PCV13 dose and one month after each PCV13 dose in the at-risk children; and just prior PCV13 vaccination, at one month post-PCV13 and at three months post-PCV13 in control children. Samples were processed at the Respiratory and Meningeal Pathogens Research Unit in Johannesburg, South Africa. Vaccine serotype-specific capsular IgG antibodies were measured using a luminex-based enzyme immunoassay as described previously [19].

### 2.2. Statistical analysis

The number of children targeted for enrolment was based on a convenience sample of children with the at-risk conditions presenting at the outpatient clinics at CHBAH, who were willing to participate in the study. Continuous variables were summarized as means or medians and compared by Student *t*-test or Wilcoxon rank-sum test between the study-groups; categorical variables were described as proportions and compared by Chi-squared test or Fisher’s exact test, as appropriate. Geometric mean concentrations (GMCs) of serotype-specific antibodies with 95% confidence intervals (95%CI) were generated and p-values between groups were computed on log<sub>10</sub> transformed data using an unpaired *t*-test. Comparisons of GMCs and fold-increase in concentration between the study-groups were performed using analysis of covariance on log<sub>10</sub> transformed data with age and baseline GMCs as covariates, where necessary. The percentage of children with GMC  $\geq 0.35$   $\mu\text{g/mL}$  (threshold measure of protective immunity against vaccine serotype IPD following the primary series in young infants) [1] and the percentage of children with  $\geq 4$ -fold-increase in antibody concentration were calculated and multivariable logistic regression was used to compare proportions between study-groups, adjusting for confounders where necessary. Data were analysed using GraphPad prism 6 (GraphPad Software, Inc. La Jolla, CA, USA) and Stata14 (StataCorp LP, Texas, USA).

### 2.3. Ethical considerations

Study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M120366) and conducted in accordance with Good Clinical Practice guidelines. Signed informed consent was obtained from the parents or legal guardians of the children for participation in this study.

## 3. Results

Overall 112 children were enrolled in the study, 45 HIV-uninfected controls with no chronic illness, 50 HIV-infected, 8 children with kidney disease and 9 with lung disease; **Table 1**. The median age at enrolment was 61.5 months, with controls having a median age of 62.3 months, which was similar to those with HIV infection (61.4 months), but higher than in children with kidney (51.6 months,  $p=0.007$ ) or lung diseases (54.1 months,  $p<0.001$ ). Overall 53.2% of participants were female and 95.5%

**Table 1**  
Demographic characteristics of the study participants.

|  | Overall          | Control participants | HIV-infected participants | Kidney disease participants | Lung disease participants |
|--|------------------|----------------------|---------------------------|-----------------------------|---------------------------|
| Number of participants (%)                           | 112              | 45 (40.2)            | 50 (45.1)                 | 8 (7.2)                     | 9 (8.1)                   |
| Female (%)   | 59 (53.2)        | 21 (47.7)            | 30 (60.0)                 | 3 (37.5)                    | 5 (55.6)                  |
| Black African descent ethnicity (%)                  | 106 (95.5)       | 42 (93.3)            | 50 (100.0)                | 6 (75.0)                    | 8 (88.9)                  |
| Visit 1: Median age - months (IQR)                   | 61.5 (55.5–63.7) | 62.3 (58.5–63.2)     | 61.4 (58.0–66.8)          | 51.6 (47.0–58.7)            | 54.1 (48.0–58.3)          |
| Visit 2: Median age - months (IQR)                   | 62.6 (56.7–64.9) | 63.3 (58.8–64.3)     | 62.5 (59.1–67.8)          | 52.6 (48.1–59.7)            | 54.2 (48.5–61.2)          |
| Visit 3: Median age - months (IQR)                   | 62.0 (54.9–67.5) | –                    | 63.6 (61.0–68.9)          | 52.2 (48.9–58.6)            | 55.2 (50.0–60.2)          |
| Last visit: Median age - months (IQR)                | 64.5 (58.8–66.4) | 65.2 (63.3–66.1)     | 64.4 (61.2–69.1)          | 53.2 (49.8–59.5)            | 56.1 (51.2–61.3)          |
| Median CD4+ cell count at enrolment - cells/mL (IQR) | –                | –                    | 1139 (814–1465)           | –                           | –                         |
| Mean CD4+ cell% at enrolment (SD)                    | –                | –                    | 32 (7.8)                  | –                           | –                         |
| Antiretroviral therapy (%)                           | –                | –                    | 49 (98)                   | –                           | –                         |
| Immunosuppressive drugs (%)                          | –                | –                    | –                         | 2 (22.2)                    | 7 (77.8)                  |

The age differences between the control versus kidney and lung disease participants were statistically significant at all visits.

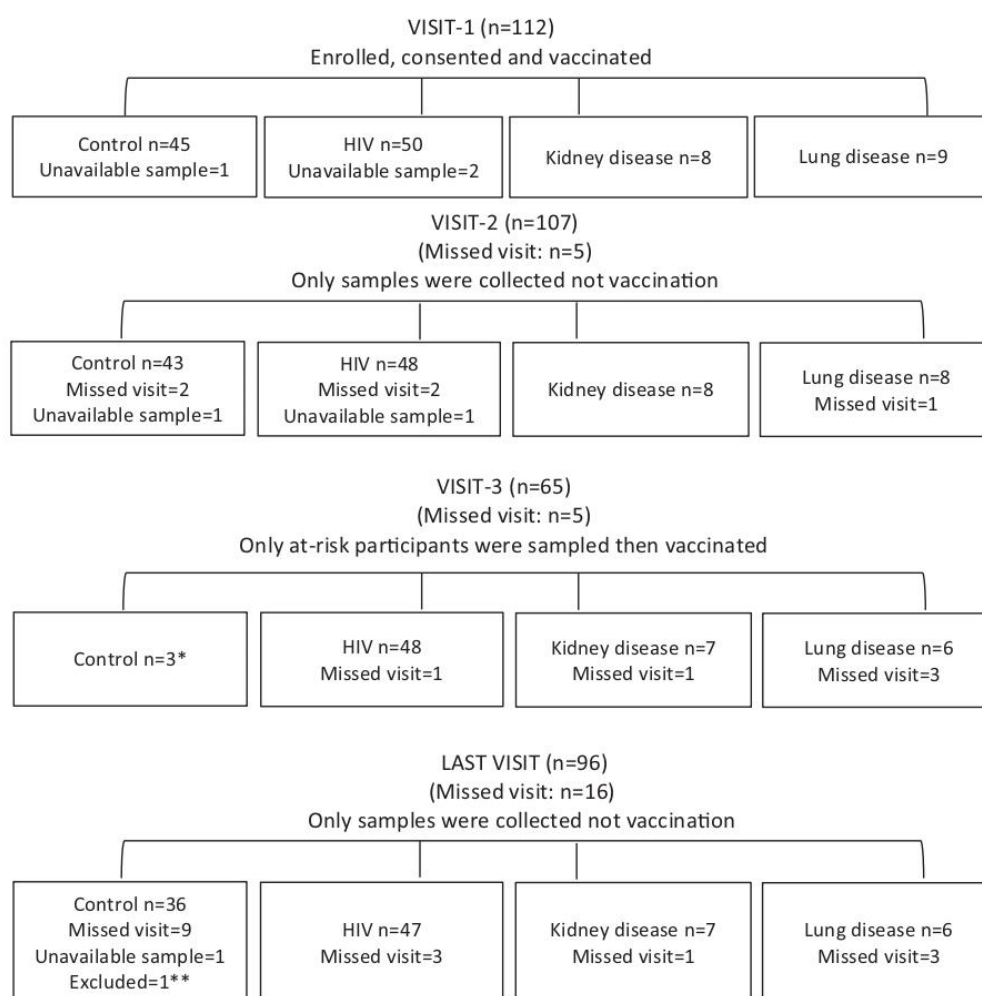
Visit-1 occurred at study enrolment, at which time children were consented into the study, baseline blood samples were taken and the first PCV13 dose was given.

Visit-2 occurred one month after enrolment, at which time blood was drawn from the children.

Visit-3 (for at-risk participants only) occurred two months after enrolment, at which time a third blood specimen was taken and the second PCV13 dose of was given.

Last visit occurred one month after the second PCV13 dose in at-risk children, and three months after the single PCV13 dose in controls.

Abbreviations: IQR: interquartile range; SD: standard deviation.



**Fig. 1.** Flowchart of participants included in the study at each visit. Some samples were either insufficient or could not be processed and were denoted as “sample unavailable”. At Visit-3, 3 control children were vaccinated by mistake, only one of these children returned for the last visit. At last study visit, the sample from a control child who was vaccinated at Visit-3 was excluded.

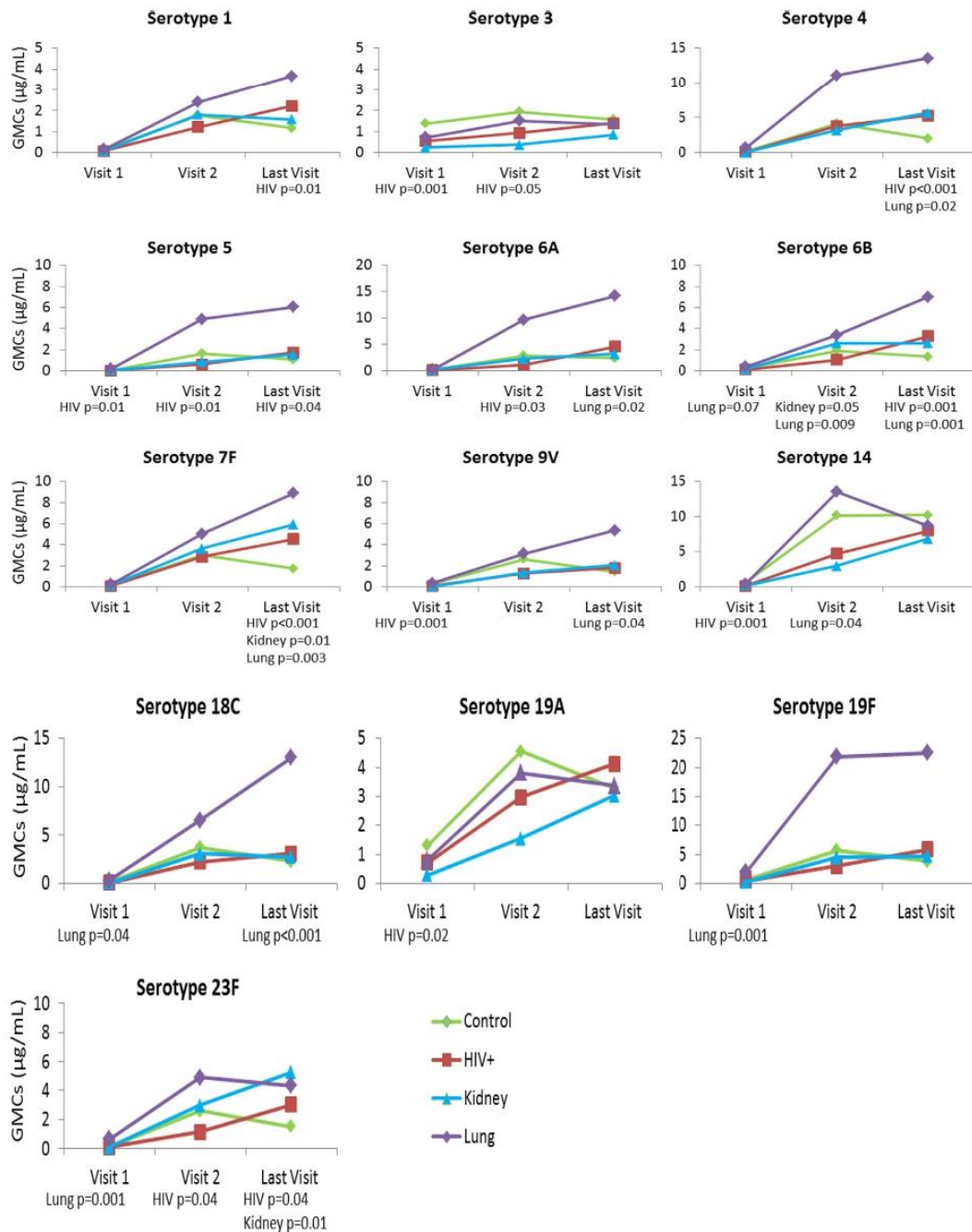
were of black African descent and this was similar between study-groups; Table 1. Among HIV-infected children 98% (n = 49) were on antiretroviral therapy (ART). The primary diagnoses of the participants with kidney disease were nephrotic syndrome (n = 4), recovering from acute kidney injury (n = 2), neonatal hypertension (n = 1) and chronic kidney disease (n = 1). At the time of study enrolment, two of the patients with nephrotic syndrome had significant proteinuria and received immunosuppressive therapy including corticosteroids and cyclophosphamide. The primary diagnoses of the participants with lung disease were asthma (n = 5), recurrent chest infections (n = 2), post-infectious chronic obstructive airways disease (n = 1) and one child had asthma and tuberculosis. The number of participants and evaluable samples at each visit is shown in Fig. 1.

### 3.1. Serotype-specific antibody concentrations at baseline

Prior to vaccination, GMCs of serotype-specific capsular antibodies in HIV-infected children were lower for five serotypes: 3

(0.6 vs. 1.4  $\mu\text{g/mL}$ ,  $p = 0.001$ ), 5 (0 vs. 0.1  $\mu\text{g/mL}$ ,  $p = 0.011$ ), 9V (0.1 vs. 0.3  $\mu\text{g/mL}$ ,  $p = 0.001$ ), 14 (0.1 vs. 0.6  $\mu\text{g/mL}$ ,  $p = 0.001$ ) and 19A (0.7 vs. 1.3  $\mu\text{g/mL}$ ,  $p = 0.017$ ), compared to control children; Fig. 2 and Supplementary Table 1. Similarly a lower percentage of HIV-infected children (4–73%) had concentration  $\geq 0.35 \mu\text{g/mL}$  at baseline compared to controls (11–93%) for all serotypes, and this was significant for serotypes 3 (58% vs. 84%,  $p = 0.011$ ), 9V (17% vs. 41%,  $p = 0.012$ ), 14 (35% vs. 61%,  $p = 0.021$ ) and 19A (73% vs. 93%,  $p = 0.007$ ); Supplementary Table 2.

Compared to controls, children with kidney disease had a trend for lower GMCs prior to vaccination for most serotypes, albeit not significant after adjusting for age; Fig. 2 and Supplementary Table 1. Thirteen to 63% of children with kidney disease had antibody concentration  $\geq 0.35 \mu\text{g/mL}$ , which was lower for seven serotypes compared to control children, however after adjusting for age only significant for serotype 3 (38% vs. 84%,  $p = 0.009$ ); Supplementary Table 2. Conversely, children with underlying lung disease generally had higher GMCs compared to controls, significantly so after adjusting for age for serotypes 6B (0.4 vs. 0.2  $\mu\text{g/mL}$ ,



**Fig. 2.** Anti-capsular geometric mean concentration for the 13 vaccine serotypes for all study-groups at Visit-1, Visit-2 and Last Visit. For each serotype, geometric mean concentrations (GMCs) are plotted for Visit-1, Visit-2 and Last Visit for each group. P-values compare each at-risk group with the control group, at each visit. P-values were adjusted for baseline antibody concentrations (all groups) and age (for the kidney and lung disease groups), and computed using an unpaired *t*-test of log<sub>10</sub> transformed antibody data, only significant (<0.05) values are shown.

$p = 0.007$ ), 18 C (0.4 vs. 0.1 µg/mL,  $p = 0.038$ ), 19 F (2.0 vs. 0.6 µg/mL,  $p = 0.001$ ) and 23 F (0.6 vs. 0.1 µg/mL,  $p = 0.001$ ); [Fig. 2](#) and [Supplementary Table 1](#). The percentage of children with concentration  $\geq 0.35$  µg/mL was also generally higher in those with

underlying lung disease (22–100%) compared to controls, including being significant for serotypes 4 (56% vs. 25%,  $p = 0.013$ ), 5 (33% vs. 11%,  $p = 0.034$ ), 19F (100% vs. 66%,  $p = 0.028$ ) and 23F (67% vs. 20%,  $p = 0.001$ ); [Supplementary Table 2](#).

### 3.2. Serotype-specific antibody concentrations one month post-first vaccination

One month post-vaccination, there was an increase in GMCs for all serotypes across all study-groups; Fig. 2. Serotype 3 and 19A had the lowest GMC fold-increases ranging from 1.4 to 2.1 and 3.4 to 5.3, respectively and the lowest percentage of children with  $\geq 4$ -fold-increase in antibody concentration ranging from 0 to 23% and 33% to 63%, respectively; Table 2. The GMC fold-increases from baseline to Visit-2 were similar in controls compared to the at-risk children for all serotypes; also, the percentage of children with  $\geq 4$ -fold-increase was similar for the controls and the three at-risk groups; Table 2. However, post-first PCV13 dose, HIV-infected children compared to controls had significantly lower GMCs for serotypes 3, 5, 6A and 23F after adjusting for baseline GMCs; Fig. 2 and Supplementary Table 1. Similarly, compared to controls and after adjusting for baseline GMCs and age, children with kidney disease generally had lower GMCs, although they exhibited a better GMCs for serotype 6B; Supplementary Table 1. Whilst those with underlying lung disease generally had higher GMCs and this was significant for serotypes 6B and 14; Fig. 2 and Supplementary Table 1.

The percentage of children with antibody concentration  $\geq 0.35\mu\text{g/mL}$  increased from baseline to one month post-vaccination in all groups, ranging from 83% to 100%, 72% to 100%, 38% to 100% and 75% to 100% for control, HIV-infected, kidney and lung disease groups respectively; Supplementary Table 2.

The percentage of children with concentration  $\geq 0.35\mu\text{g/mL}$  was similar in the at-risk and control children except that a lower percentage of HIV-infected children (80% vs. 100%,  $p = 0.031$ ) and children with kidney disease (38% vs. 100%,  $p = 0.003$ ) achieved that threshold for serotype 3; Supplementary Table 2. All of the children with lung disease had concentration  $\geq 0.35\mu\text{g/mL}$  for 11 serotypes, and most had antibody concentrations above this threshold for serotypes 3 (75%) and 6B (88%); Supplementary Table 2.

### 3.3. Serotype-specific antibody concentrations three months post-first vaccination

At the last study-visit, three months post-first PCV13 dose, and one month post-second PCV13 dose for the at-risk groups, GMCs were generally higher in the at-risk groups compared to the controls for all serotypes, except serotypes 3 and 14, which were higher in the control children compared to all at-risk groups, and serotype 19A that was higher in the controls compared to children with kidney disease, albeit these differences were not significant; Fig. 2, Supplementary Table 1. HIV-infected children had significantly higher GMCs compared to the controls for serotypes 1, 4, 5, 6B, 7F and 23F after adjusting for baseline GMCs. After adjusting for baseline GMCs and age, children with kidney disease achieved significantly higher GMCs than controls only for serotypes 7F and 23F; while higher GMCs were observed in children with lung disease for serotypes 4, 6A, 6B, 7F, 9V and 18C compared to controls; Fig. 2, Supplementary Table 1.

**Table 2**

Fold-increase in serotype-specific antibody geometric mean concentration and percentage of children with at least 4-fold-increase in antibody concentration between baseline and Visit-2.

| Fold-increase between baseline and Visit-2   |                  |                  |       |                  |                |                  |                |
|--|------------------|------------------|-------|------------------|----------------|------------------|----------------|
| Serotype   | Control (n = 42) | HIV (n = 44)     | p     | Kidney (n = 8)   | p <sup>*</sup> | Lung (n = 8)     | p <sup>*</sup> |
| 1  | 12.3 (7.6–19.9)  | 15.1 (9.1–25.0)  | 0.554 | 21.6 (3.3–142.8) | 0.908          | 17.0 (4.0–72.7)  | 0.657          |
| 3  | 1.4 (1.0–1.9)    | 1.8 (1.1–2.9)    | 0.386 | 1.5 (0.9–2.6)    | 0.565          | 2.1 (1.2–3.6)    | 0.888          |
| 4  | 26.0 (14.9–45.5) | 44.2 (22.6–86.4) | 0.225 | 84.5 (8.7–821.0) | 0.478          | 14.1 (3.8–52.0)  | 0.631          |
| 5  | 25.8 (15.8–42.3) | 24.2 (13.7–42.7) | 0.862 | 16.9 (3.4–83.2)  | 0.356          | 25.8 (7.4–90.0)  | 0.734          |
| 6A   | 16.5 (10.2–26.8) | 10.5 (5.9–18.9)  | 0.236 | 11.8 (3.2–43.6)  | 0.411          | 33.1 (4.7–231.2) | 0.314          |
| 6B   | 8.9 (5.7–13.8)   | 9.1 (4.7–17.5)   | 0.950 | 19.1 (5.9–62.2)  | 0.058          | 7.9 (2.8–22.0)   | 0.579          |
| 7F   | 18.0 (11.8–27.5) | 27.1 (17.1–43.0) | 0.191 | 27.0 (3.1–234.8) | 0.679          | 17.5 (5.0–60.6)  | 0.503          |
| 9V   | 8.6 (5.0–14.9)   | 12.9 (7.3–23.0)  | 0.303 | 26.1 (4.6–149.8) | 0.550          | 7.7 (3.2–18.5)   | 0.694          |
| 14   | 16.0 (8.6–29.8)  | 42.9 (18.6–98.9) | 0.062 | 19.1 (3.0–120.2) | 0.901          | 33.3 (4.2–263.6) | 0.747          |
| 18C  | 23.9 (11.4–50.1) | 28.5 (13.8–58.8) | 0.730 | 33.8 (4.2–273.5) | 0.796          | 18.3 (2.2–149.5) | 0.273          |
| 19A  | 3.4 (2.3–4.9)    | 3.8 (2.1–6.6)    | 0.746 | 5.3 (2.5–11.1)   | 0.202          | 5.0 (2.7–9.4)    | 0.281          |
| 19F  | 9.3 (5.8–14.9)   | 6.2 (3.6–10.5)   | 0.253 | 16.3 (2.3–115.2) | 0.665          | 10.9 (2.6–46.1)  | 0.971          |
| 23F  | 18.8 (11.4–30.9) | 15.1 (8.1–28.1)  | 0.585 | 29.3 (8.4–102.0) | 0.179          | 8.5 (3.8–19.1)   | 0.435          |
| Percentage of participants with $\geq 4$ -fold-increase between baseline and Visit-2 |                  |                  |       |                  |                |                  |                |
| Serotype   | Control n = (42) | HIV (n = 47)     | p     | Kidney (n = 8)   | p <sup>*</sup> | Lung (n = 8)     | p <sup>*</sup> |
| 1  | 33; 79% (70–90)  | 36; 82% (70–90)  | 0.790 | 6; 75% (40–110)  | 0.614          | 7; 88% (60–120)  | 0.775          |
| 3  | 4; 10% (0–20)    | 10; 23% (10–40)  | 0.144 | 0; 0%            | 1.000          | 1; 13% (-20–40)  | 0.637          |
| 4  | 37; 88% (80–100) | 39; 89% (80–110) | 1.000 | 7; 88% (60–120)  | 0.836          | 5; 63% (20–110)  | 0.062          |
| 5  | 39; 93% (80–100) | 38; 86% (80–100) | 0.485 | 7; 88% (60–120)  | 0.490          | 6; 75% (40–110)  | 0.090          |
| 6A   | 33; 79% (70–90)  | 31; 70% (60–80)  | 0.463 | 5; 63% (20–110)  | 0.304          | 6; 75% (40–110)  | 0.751          |
| 6B   | 31; 74% (60–90)  | 31; 70% (60–80)  | 0.812 | 7; 88% (60–120)  | 0.368          | 5; 63% (20–110)  | 0.643          |
| 7F   | 37; 88% (80–100) | 41; 93% (90–100) | 0.479 | 6; 75% (40–110)  | 0.179          | 6; 75% (40–110)  | 0.135          |
| 9V   | 28; 67% (50–80)  | 33; 75% (60–90)  | 0.479 | 8; 100% (60–120) | 0.657          | 5; 63% (20–110)  | 0.560          |
| 14   | 32; 76% (60–90)  | 37; 84% (70–100) | 0.423 | 5; 63% (20–110)  | 0.268          | 6; 75% (40–110)  | 0.667          |
| 18C  | 35; 83% (70–100) | 36; 82% (70–90)  | 1.000 | 6; 75% (40–110)  | 0.327          | 5; 63% (20–110)  | 0.061          |
| 19A  | 14; 33% (20–50)  | 20; 45% (30–60)  | 0.277 | 5; 63% (20–110)  | 0.289          | 4; 50% (10–90)   | 0.699          |
| 19F  | 28; 67% (50–80)  | 24; 55% (40–80)  | 0.228 | 5; 63% (20–110)  | 0.517          | 6; 75% (40–110)  | 0.991          |
| 23F  | 35; 83% (70–100) | 34; 77% (60–90)  | 0.591 | 8; 100%          | 0.674          | 6; 75% (40–110)  | 0.563          |

For each serotype, geometric mean concentration (GMC) fold-increases are reported with 95% confidence intervals in brackets, and the proportion of children with  $\geq 4$ -fold-increase are reported as number of observations, percentage and percentage confidence intervals in brackets.

P-values were computed using an unpaired t-test and ANOVA on log10 transformed antibody data for GMCs and Chi-squared or Fisher's exact tests and logistic regression for percentages.

p: p-value comparing HIV-infected children with the control group, after adjusting for baseline GMCs.

p<sup>\*</sup>: p-value comparing each at-risk group with the control group, after adjusting for baseline GMCs and age (for the kidney and lung disease groups).

HIV: HIV-infected children; Kidney: children with kidney disease; Lung: children with lung disease.

**Table 3**

Fold-increase in serotype-specific antibody geometric mean concentration and percentage of children with at least 4-fold-increase in antibody concentration between baseline and Visit-2 and between baseline and final visit in the at-risk groups.

| Serotype   | HIV (n = 44)<br>Visit-2/Visit-1 | HIV (n = 45)<br>Last visit/Visit-1 | p                | Kidney (n = 8)<br>Visit-2/Visit-1 | Kidney (n = 7)<br>Last visit/Visit-1 | p     | Lung (n = 8)<br>Visit-2/Visit-1 | Lung (n = 6)<br>Last visit/Visit-1 | p            |
|--|---------------------------------|------------------------------------|------------------|-----------------------------------|--------------------------------------|-------|---------------------------------|------------------------------------|--------------|
| <i>Fold-increase between Visit-1 and Visit-2 and between Visit-1 and final visit for the at-risk groups</i>                                |                                 |                                    |                  |                                   |                                      |       |                                 |                                    |              |
| 1  | 15.1 (9.1–25.0)                 | 27.6 (16.9–44.9)                   | <b>&lt;0.001</b> | 21.6 (3.3–142.8)                  | 27.0 (3.9–187.8)                     | 0.693 | 17.0 (4.0–72.7)                 | 42.8 (6.3–292.7)                   | 0.545        |
| 3  | 1.8 (1.1–2.9)                   | 2.6 (1.7–4.0)                      | <b>0.007</b>     | 1.5 (0.9–2.6)                     | 3.3 (1.6–6.5)                        | 0.052 | 2.1 (1.2–3.6)                   | 2.5 (0.7–9.2)                      | 0.687        |
| 4  | 44.2 (22.6–86.4)                | 60.4 (34.1–107.0)                  | 0.086            | 84.5 (8.7–821.0)                  | 255.6 (14.3–4571.3)                  | 0.532 | 14.1 (3.8–52.0)                 | 27.9 (4.9–159.5)                   | 0.663        |
| 5  | 24.2 (13.7–42.7)                | 70.7 (41.2–121.4)                  | <b>&lt;0.001</b> | 16.9 (3.4–83.2)                   | 48.1 (6.8–340.6)                     | 0.343 | 25.8 (7.4–90.0)                 | 79.3 (60.3–104.4)                  | 0.308        |
| 6A   | 10.5 (5.9–18.9)                 | 46.7 (25.5–85.5)                   | <b>&lt;0.001</b> | 11.8 (3.2–43.6)                   | 28.1 (4.3–181.8)                     | 0.273 | 33.1 (4.7–231.2)                | 125.2 (21.5–730.1)                 | 0.411        |
| 6B   | 9.1 (4.7–17.5)                  | 25.8 (14.2–46.6)                   | <b>&lt;0.001</b> | 19.1 (5.9–62.2)                   | 23.2 (3.6–151.4)                     | 0.954 | 7.9 (2.8–22.0)                  | 27.4 (8.8–85.4)                    | 0.245        |
| 7F   | 27.1 (17.1–43.0)                | 45.5 (27.7–74.8)                   | <b>&lt;0.001</b> | 27.0 (3.1–234.8)                  | 69.5 (9.7–497.2)                     | 0.722 | 17.5 (5.0–60.6)                 | 72.0 (19.0–272.6)                  | 0.143        |
| 9V   | 12.9 (7.3–23.0)                 | 17.7 (10.5–30.1)                   | 0.051            | 26.1 (4.6–149.8)                  | 47.3 (8.6–261.4)                     | 0.635 | 7.7 (3.2–18.5)                  | 25.5 (9.3–70.2)                    | <b>0.026</b> |
| 14   | 42.9 (18.6–98.9)                | 72.8 (35.2–150.8)                  | <b>0.020</b>     | 19.1 (3.0–120.2)                  | 61.9 (5.7–671.4)                     | 0.140 | 33.3 (4.2–263.6)                | 93.4 (16.9–516.1)                  | 0.710        |
| 18C  | 28.5 (13.8–58.8)                | 36.6 (18.3–73.0)                   | 0.135            | 33.8 (4.2–273.5)                  | 40.3 (4.1–394.2)                     | 0.626 | 18.3 (2.2–149.5)                | 79.7 (7.5–843.6)                   | 0.156        |
| 19A  | 3.8 (2.1–6.6)                   | 5.3 (3.2–8.7)                      | 0.050            | 5.3 (2.5–11.1)                    | 10.9 (4.4–26.8)                      | 0.125 | 5.0 (2.7–9.4)                   | 6.1 (1.8–20.3)                     | 0.894        |
| 19F  | 6.2 (3.6–10.5)                  | 13.0 (7.7–21.9)                    | <b>&lt;0.001</b> | 16.3 (2.3–115.2)                  | 25.2 (2.9–217.1)                     | 0.891 | 10.9 (2.6–46.1)                 | 17.6 (2.0–152.6)                   | 0.890        |
| 23F  | 15.1 (8.1–28.1)                 | 38.9 (19.9–75.8)                   | <b>&lt;0.001</b> | 29.3 (8.4–102.0)                  | 89.5 (21.0–382.1)                    | 0.277 | 8.5 (3.8–19.1)                  | 17.4 (5.7–52.8)                    | 0.390        |
| <i>Percentage of participants with ≥4-fold-increase between Visit-1 and Visit-2 and between Visit-1 and final visit for at-risk groups</i> |                                 |                                    |                  |                                   |                                      |       |                                 |                                    |              |
| 1  | 36; 82% (70–90)                 | 40; 89% (80–100)                   | 0.384            | 6; 75% (40–110)                   | 5; 71% (30–120)                      | 1.000 | 7; 88% (60–120)                 | 5; 83% (40–130)                    | 1.000        |
| 3  | 10; 23% (10–40)                 | 18; 40% (30–50)                    | 0.110            | 0; 0% (0–0)                       | 3; 43% (-10–90)                      | 0.077 | 1; 13% (-20–40)                 | 2; 33% (-20–90)                    | 0.539        |
| 4  | 39; 89% (80–110)                | 41; 91% (80–100)                   | 0.739            | 7; 88% (60–120)                   | 6; 86% (50–120)                      | 1.000 | 5; 63% (20–110)                 | 5; 83% (40–130)                    | 0.580        |
| 5  | 38; 86% (80–100)                | 42; 93% (90–100)                   | 0.315            | 7; 88% (60–120)                   | 6; 86% (50–120)                      | 1.000 | 6; 75% (40–110)                 | 6; 100% (100–100)                  | 0.473        |
| 6A   | 31; 70% (60–80)                 | 41; 91% (80–100)                   | <b>0.016</b>     | 5; 63% (20–110)                   | 5; 71% (30–120)                      | 1.000 | 6; 75% (40–110)                 | 6; 100% (100–100)                  | 0.473        |
| 6B   | 31; 70% (60–80)                 | 35; 78% (70–90)                    | 0.475            | 7; 88% (60–120)                   | 6; 86% (50–120)                      | 1.000 | 5; 63% (20–110)                 | 6; 100% (100–100)                  | 0.209        |
| 7F   | 41; 93% (90–100)                | 43; 96% (90–100)                   | 0.677            | 6; 75% (40–110)                   | 6; 86% (50–120)                      | 1.000 | 6; 75% (40–110)                 | 6; 100% (100–100)                  | 0.473        |
| 9V   | 33; 75% (60–90)                 | 36; 80% (70–90)                    | 0.619            | 8; 100% (60–120)                  | 6; 86% (50–120)                      | 0.467 | 5; 63% (20–110)                 | 6; 100% (100–100)                  | 0.209        |
| 14   | 37; 84% (70–100)                | 40; 89% (80–100)                   | 0.550            | 5; 63% (20–110)                   | 6; 86% (50–120)                      | 0.569 | 6; 75% (40–110)                 | 6; 100% (100–100)                  | 0.473        |
| 18C  | 36; 82% (70–90)                 | 39; 87% (80–100)                   | 0.573            | 6; 75% (40–110)                   | 6; 86% (50–120)                      | 1.000 | 5; 63% (20–110)                 | 5; 83% (40–130)                    | 0.580        |
| 19A  | 20; 45% (30–60)                 | 27; 60% (50–70)                    | 0.205            | 5; 63% (20–110)                   | 7; 100% (100–100)                    | 0.200 | 4; 50% (10–90)                  | 4; 67% (10–120)                    | 0.627        |
| 19F  | 24; 55% (40–80)                 | 34; 76% (60–90)                    | <b>0.047</b>     | 5; 63% (20–110)                   | 6; 86% (50–120)                      | 0.569 | 6; 75% (40–110)                 | 4; 67% (10–120)                    | 1.000        |
| 23F  | 34; 77% (60–90)                 | 37; 82% (70–90)                    | 0.606            | 8; 100% (100–100)                 | 7; 100% (100–100)                    | 1.000 | 6; 75% (40–110)                 | 6; 100% (100–100)                  | 0.473        |

For each serotype, geometric mean concentration (GMC) fold-increases are reported with 95% confidence intervals in brackets, and the proportion of children with ≥4-fold-increase are reported as number of observations, percentage and percentage confidence intervals in brackets.

P-values were computed using an unpaired t-test on log10 transformed antibody data for GMCs and Chi-squared or Fisher's exact tests for percentages.

P-values in bold are <0.05 and taken as statistically significant.

p: p-value comparing fold-increase between Visit-2 and Visit-1 and fold-increase between final visit and Visit-1, for each at-risk group.

HIV: HIV-infected children; Kidney: children with kidney disease; Lung: children with lung disease.

### 3.4. Increase in antibody concentrations from baseline to after first and second PCV13 doses for at-risk groups

The fold-increases in GMCs from baseline to post-second PCV13 dose compared to fold-increases from baseline to post-first dose in HIV-infected children were significantly higher for all but four serotypes, namely 4, 9V, 18C and 19A. Nonetheless the percentage of HIV-infected children with ≥4-fold-increase in antibody concentration from baseline were similar after the second PCV13 dose compared to post-first dose for all serotypes except 6A and 19F; **Table 3**. After the second PCV13 dose >90% of HIV-infected children achieved concentrations ≥0.35 µg/mL for all serotypes except serotype 23F (87%) and a higher percentage of children had concentrations ≥0.35 µg/mL compared to post-first dose for four serotypes (5, 6A, 6B and 18C); **Table 4**.

In children with kidney or lung disease the GMC fold-increases from baseline to post-second vaccine dose compared to fold-increases from baseline to post-first dose were similar for all serotypes, except for being higher for serotype 9V in children with lung disease; **Table 3**. After second vaccination the percentage of children with concentrations ≥0.35 µg/mL in the kidney disease group was ≥86% except for serotype 3 (71%), and in the lung disease group was 100% for all serotypes except for serotype 3 (83%); and these percentages were similar to post-first PCV13 dose; **Table 4**.

## 4. Discussion

Our study focused on the immunological responses to PCV13 in children approximately 5 years old with underlying medical condi-

tions. PCV13 is generally recommended for children 2–23 months of age; however, after the introduction of PCV13 in South Africa, a catch-up program was implemented in older children 18–36 months of age, and children 36–71 months of age with underlying medical conditions, including those infected with HIV, and those with kidney and lung diseases [17]. A good humoral response was detected in all at-risk groups post-first and second PCV13 vaccinations, for all serotypes except for serotypes 3 and 19A. After first vaccine dose, a similar percentage of controls and at-risk children achieved a ≥4-fold-increase in antibody concentrations and had, in general, concentrations ≥0.35 µg/mL. Still, at every visit, the at-risk children had lower GMCs for serotypes 3 and 14 (except the lung disease group post-first PCV13 for serotype 14), compared to control children.

Previous studies in younger children, had found that compared to HIV-uninfected children HIV-infected have inferior responses to PCVs in the absence of effective ART, including lower serotype-specific GMCs, lower percentage achieving concentrations ≥0.35 µg/mL post-PCV and lower opsonophagocytic assay (OPA) responses [20–22]. HIV-infected children with CD4+ cell percentages >25% and early initiation of ART have nonetheless been demonstrated to have mostly similar PCV responses compared to HIV-uninfected children [20–22], as in the present study in which 98% of HIV-infected participants were on ART at enrolment. HIV-infected children generally had good responses to PCV13 compared to control children, with similar percentage of children having a ≥4-fold-increase from baseline to post-first PCV13 dose and similar percentage with antibody concentration ≥0.35 µg/mL, except for serotype 3; GMCs were, however, lower for four of the 13 serotypes. While an increase in GMCs was observed in these children



**Table 4**Percentage of children with antibody concentration  $\geq 0.35$   $\mu\text{g/mL}$  at Visit-2 and final visit in the at-risk groups.

| Serotype | HIV (n = 46)<br>Visit-2 | HIV (n = 47)<br>Last visit | p            | Kidney (n = 8)<br>Visit-2 | Kidney (n = 7)<br>Last visit | p     | Lung (n = 8)<br>Visit-2 | Lung (n = 6)<br>Last visit | p     |
|----------|-------------------------|----------------------------|--------------|---------------------------|------------------------------|-------|-------------------------|----------------------------|-------|
| 1        | 38; 83% (69–91)         | 44; 94% (82–98)            | 0.120        | 8; 100% (63–100)          | 6; 86% (47–99)               | 0.467 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 3        | 37; 80% (67–90)         | 44; 94% (82–98)            | 0.070        | 3; 38% (13–70)            | 5; 71% (35–92)               | 0.667 | 6; 75% (40–94)          | 5; 83% (42–99)             | 1.000 |
| 4        | 42; 91% (79–97)         | 47; 100% (91–100)          | 0.056        | 8; 100% (63–100)          | 6; 86% (47–99)               | 0.467 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 5        | 34; 74% (60–85)         | 43; 91% (80–97)            | <b>0.030</b> | 6; 75% (40–94)            | 6; 86% (47–99)               | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 6A       | 35; 76% (62–86)         | 44; 94% (82–98)            | <b>0.022</b> | 7; 88% (51–100)           | 6; 86% (47–99)               | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 6B       | 33; 72% (57–83)         | 43; 91% (80–97)            | <b>0.017</b> | 7; 88% (51–100)           | 6; 86% (47–99)               | 1.000 | 7; 88% (51–100)         | 6; 100% (56–100)           | 1.000 |
| 7F       | 46; 100% (91–100)       | 47; 100% (91–100)          | 1.000        | 8; 100% (63–100)          | 6; 86% (47–99)               | 0.467 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 9V       | 36; 78% (64–88)         | 43; 91% (80–97)            | 0.089        | 6; 75% (40–94)            | 6; 86% (47–99)               | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 14       | 43; 93% (82–98)         | 47; 100% (91–100)          | 0.117        | 8; 100% (63–100)          | 7; 100% (60–100)             | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 18C      | 40; 87% (74–94)         | 47; 100% (91–100)          | <b>0.012</b> | 8; 100% (63–100)          | 6; 86% (47–99)               | 0.467 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 19A      | 41; 89% (77–96)         | 46; 98% (88–100)           | 0.111        | 7; 88% (51–100)           | 7; 100% (60–100)             | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 19F      | 43; 93% (82–98)         | 46; 98% (88–100)           | 0.361        | 7; 88% (51–100)           | 7; 100% (60–100)             | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |
| 23F      | 36; 78% (64–88)         | 41; 87% (74–94)            | 0.284        | 7; 88% (51–100)           | 6; 86% (47–99)               | 1.000 | 8; 100% (63–100)        | 6; 100% (56–100)           | 1.000 |

For each serotype, the percentage of children with antibody concentration  $\geq 0.35$   $\mu\text{g/mL}$  is reported as number of observations, percentage and percentage confidence intervals in brackets.

P-values were computed using Chi-squared or Fisher's exact tests and logistic regression.

P-values in bold are  $< 0.05$  and taken as statistically significant.

HIV: HIV-infected children; Kidney: children with kidney disease; Lung: children with lung disease.

after the second PCV13 dose for nine serotypes, the percentage of children having a  $\geq 4$ -fold-increase from baseline or achieving concentrations  $\geq 0.35$   $\mu\text{g/mL}$  was similar to post-first dose except for two and four serotypes respectively.

Children with kidney disease also had good responses to PCV13 post-first dose, with GMC fold-increases and percentage with  $\geq 4$ -fold-increase from baseline similar to that of control children. These children had conspicuously low responses to serotype 3, relative to the other serotypes, with none of the children achieving  $\geq 4$ -fold-increase post-first vaccine dose. These children are at increased risk for severe pneumococcal infections, with defective IgG synthesis observed in those on steroidal therapy [23]. PCV7 was found to be immunogenic in children with idiopathic nephrotic syndrome in remission, although the magnitude and persistence of response to some serotypes was inferior compared with healthy subjects, specifically inferior immunogenicity for three of the seven serotypes was observed in patients on immunomodulators [24]. In our study, only 22% of these children were on immunosuppressive drugs, which may explain their better responses to PCV13. After the second PCV13 dose, both GMC fold-increase and percentage with  $\geq 4$ -fold-increase from baseline were similar to those observed post-first dose, suggesting that the second dose did not have an effect.

Surprisingly, children with underlying lung disease not only had very good responses to PCV13 that were mostly similar to the control children, but their GMCs were higher for all but serotype 3, and 100% of these children achieved antibody concentration  $\geq 0.35$   $\mu\text{g/mL}$  after first PCV13 dose for all serotypes except serotype 3 (75%) and 6B (88%). Due to their underlying lung disease, these children may be more susceptible to pneumococcal infections, and therefore may have had previous pneumococcal infections, resulting in priming by natural infection. These children also did not show a benefit from a second PCV13 dose.

Relative to the other serotypes, serotypes 3 and 19A induced inferior responses in all study-groups, but especially so in the at-risk groups. After vaccination, GMC fold-increases were noticeably lower for serotypes 3 and 19A, ranging from 1.4 to 2.1 and 1.1 to 3.3 for serotype 3 after first and second dose respectively, and 3.4 to 5.3 after first and 2.2 to 10.9 after second dose for serotype 19A. Furthermore, in none of the at-risk groups did 100% of children achieve a concentration of  $\geq 0.35$   $\mu\text{g/mL}$  for serotype 3. In children with lung disease, despite high GMCs and 100% of children with concentrations  $\geq 0.35$   $\mu\text{g/mL}$  post-first PCV13 dose for 11 of 13 serotypes, serotype 3 responses were lower compared

to controls and only 83% had concentrations  $\geq 0.35$   $\mu\text{g/mL}$ , even post-second dose. Also, only 38% and 71% of children with kidney disease achieved levels  $\geq 0.35$   $\mu\text{g/mL}$  for serotype 3 after first and second PCV13 dose, respectively. This hyporesponse to serotype 3 has been described in several immunogenicity studies, even after a booster dose of vaccine [25–31]. Furthermore, in the development of a 10-valent PCV (PCV10), serotype 3 was excluded from the formulation, due to absence of efficacy and anamnestic responses in the earlier investigational 11-valent formulation [32]. Serotype 3 causes invasive disease mainly in older children and adults, however, it has been suggested that the use of insensitive culture methods might underestimate its role on paediatric IPD [33]. The lower immunogenicity of serotype 3 can partially be attributed to its immune evasion mechanism of having a larger and more mucoid polysaccharide capsule [34], which inhibits phagocytosis by immune cells [35,36]. Also, while GMCs for serotype 14 were reasonably high, those of the at-risk groups were lower compared to the controls at all visits, except Visit-2 in the lung disease children. The lower response to these serotypes in at-risk groups warrants further study.

A limitation to our study was that at-risk children were enrolled based on a convenience sample of children presenting at the outpatient clinics, thus for children with kidney and lung disease a small sample size of 8 and 9 respectively was used in this analysis, limiting the power of the statistical comparisons. Furthermore since we did not measure responses by OPA, no antibody functionality was assessed [37]. Another limitation was the use of the 0.35  $\mu\text{g/mL}$  threshold, reported as the antibody concentration which protects against IPD in young healthy children following the PCV primary series [1]. While this value has been used extensively, its applicability as a correlate of protection has not been established for children older than 15 months and those with underlying medical conditions. In fact, studies have indicated that levels  $> 0.35$   $\mu\text{g/mL}$  may be required to protect at-risk groups from IPD and that this threshold may not be predictive of protection across all PCV13 serotypes [28,38,39]. Nonetheless the relation between serotype-specific antibody concentration and long-term protection needs to be better characterized since the durability of protection may differ in children with chronic diseases compared to healthy children.

In conclusion, children with underlying conditions, namely HIV-infection, kidney and lung disease had similar responses to PCV13 compared to control children, with exception of a few serotypes. A good immune response was observed for all but serotypes 3 and

19A, in all study-groups. Responses after a second PCV13 dose in at-risk older, vaccine-naïve children with kidney and lung disease suggest that a single vaccine dose might be sufficient to trigger an adequate antibody response. PCV-naïve, older HIV-infected children may benefit from two doses of PCV13. In future studies a larger spectrum of comorbidities should be included to determine the most cost effective vaccination regimens particularly in low income countries.

### Conflict of interest

SAM has received grant support from Pfizer and has received honoraria for serving on Pfizer speaker bureaus and advisory boards. MCN has received honoraria from Pfizer.

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### Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vaccine.2017.06.081>.

### References

- [1] Pneumococcal conjugate vaccine for childhood immunisation-World Health Organization position paper. *Wkly Epidemiol Rec* 2007;82:93–104.
- [2] Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Pierce N. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N Engl J Med* 2003;349(14):1341–8.
- [3] Conklin L, Loo JD, Kirk J, Fleming-Dutra KE, Deloria Knoll M, Park DE, et al. Systematic review of the effect of pneumococcal conjugate vaccine dosing schedules on vaccine-type invasive pneumococcal disease among young children. *Pediatr Infect Dis J* 2014;33(Suppl 2):S109–18.
- [4] Direct and indirect effects of routine vaccination of children with 7-valent pneumococcal conjugate vaccine on incidence of invasive pneumococcal disease—United States, 1998–2003. Centers for Disease Control and Prevention (CDC). Morbidity and mortality weekly report. 2005;54:893–7.
- [5] O'Brien KL, Moulton LH, Reid R, Weatherholtz R, Oski J, Brown L, et al. Efficacy and safety of seven-valent conjugate pneumococcal vaccine in American Indian children: group randomised trial. *Lancet* 2003;362(9381):355–61.
- [6] Black SB, Shinefield HR, Hansen J, Elvin L, Laufer D, Malinoski F. Postlicensure evaluation of the effectiveness of seven valent pneumococcal conjugate vaccine. *Pediatr Infect Dis J* 2001;20(12):1105–7.
- [7] Pilishvili T, Lexas C, Farley MM, Hadler J, Harrison LH, Bennett NM, et al. Sustained reductions in invasive pneumococcal disease in the era of conjugate vaccine. *J Infect Dis* 2010;201(1):32–41.
- [8] Cutts FT, Zaman SM, Enwere G, Jaffar S, Levine OS, Okoko JB, et al. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. *Lancet* 2005;365(9465):1139–46.
- [9] Johnson HL, Deloria-Knoll M, Levine OS, Stoszek SK, Freimanis Hance L, Reithinger R, et al. Systematic evaluation of serotypes causing invasive pneumococcal disease among children under five: the pneumococcal global serotype project. *PLoS Med* 2010;7(10).
- [10] Aguiar SI, Brito MJ, Goncalo-Marques J, Melo-Cristino J, Ramirez M. Serotypes 1, 7F and 19A became the leading causes of pediatric invasive pneumococcal infections in Portugal after 7 years of heptavalent conjugate vaccine use. *Vaccine* 2010;28(32):5167–73.
- [11] Feikin DR, Kagucia EW, Loo JD, Link-Gelles R, Puhon MA, Cherian T, et al. Serotype-specific changes in invasive pneumococcal disease after pneumococcal conjugate vaccine introduction: a pooled analysis of multiple surveillance sites. *PLoS Med* 2013;10(9):e1001517.
- [12] Mistry RD, Wedin T, Balamuth F, McGowan KL, Ellison AM, Nelson KA, et al. Emergency department epidemiology of pneumococcal bacteremia in children since the institution of widespread PCV7 vaccination. *J Emerg Med* 2013;45(6):813–20.
- [13] Jefferies JM, Macdonald E, Faust SN, Clarke SC. 13-valent pneumococcal conjugate vaccine (PCV13). *Hum Vaccin* 2011;7(10):1012–8.
- [14] Cohen C, von Mollendorf C, de Gouveia L, Lengana S, Meiring S, Quan V, et al. Effectiveness of the 13-valent pneumococcal conjugate vaccine against invasive pneumococcal disease in South African children: a case-control study. *Lancet Glob Health* 2017.
- [15] Respiratory and Meningeal Pathogens Reference Unit, National Institute for Communicable Diseases. GERM5-SA surveillance report: Streptococcus pneumoniae. At: <<http://www.nicd.ac.za/assets/files/Bulletin%20May%202011.pdf>> (accessed Dec 8, 2016). *Communicable diseases surveillance bulletin*. May, 2011;9(2):50–3.
- [16] von Mollendorf C, Cohen C, Tempia S, Meiring S, de Gouveia L, Quan V, et al. Epidemiology of serotype 1 invasive pneumococcal disease, South Africa, 2003–2013. *Emerg Infect Dis* 2016;22(2):261–70.
- [17] Centers for Disease Control. Preventing pneumococcal disease among infants and young children. Recommendations of the advisory committee on immunization practices (ACIP). *MMWR Recomm Rep* 2000;49(RR-9):1–35.
- [18] Nunes MC, Madhi SA. Pneumococcal conjugate vaccine in immunocompromised individuals. *Pneumococcal conjugate vaccines: Future Medicine Ltd*; March 2012. p. 46–62.
- [19] Madhi SA, Adrian P, Cotton MF, McIntyre JA, Jean-Philippe P, Meadows S, et al. Effect of HIV infection status and anti-retroviral treatment on quantitative and qualitative antibody responses to pneumococcal conjugate vaccine in infants. *J Infect Dis* 2010;202(3):355–61.
- [20] King Jr JC, Vink PE, Chang I, Kimura A, Parks M, Smilie M, et al. Antibody titers eight months after three doses of a five-valent pneumococcal conjugate vaccine in HIV and non-HIV-infected children less than two years of age. *Vaccine* 1998;16(4):361–5.
- [21] Madhi SA, Kuwanda L, Cutland C, Holm A, Kayhty H, Klugman KP. Quantitative and qualitative antibody response to pneumococcal conjugate vaccine among African human immunodeficiency virus-infected and uninfected children. *Pediatr Infect Dis J* 2005;24(5):410–6.
- [22] Spoulou VI, Tsoumas DL, Papaevangelou VG, Mostrou GI, Theodoridou MC. Immunogenicity and immunological memory induced by a 7-valent pneumococcal CRM197 conjugate vaccine in symptomatic HIV-1 infected children. *Vaccine* 2005;23(46–47):5289–93.
- [23] Heslan JM, Lautie JP, Intrator L, Blanc C, Lagrue G, Sobel AT. Impaired IgG synthesis in patients with the nephrotic syndrome. *Clin Nephrol* 1982;18(3):144–7.
- [24] Liakou CD, Askiti V, Mitsioni A, Stefanidis CJ, Theodoridou MC, Spoulou VI. Safety, immunogenicity and kinetics of immune response to 7-valent pneumococcal conjugate vaccine in children with idiopathic nephrotic syndrome. *Vaccine* 2011;29(40):6834–7.
- [25] Dagan R, Juergens C, Trammel J, Patterson S, Greenberg D, Givon-Lavi N, et al. Efficacy of 13-valent pneumococcal conjugate vaccine (PCV13) versus that of 7-valent PCV (PCV7) against nasopharyngeal colonization of antibiotic-nonsusceptible *Streptococcus pneumoniae*. *J Infect Dis* 2014;211(7):1144–53.
- [26] Poolman J, Borrow R. Hyporesponsiveness and its clinical implications after vaccination with polysaccharide or glycoconjugate vaccines. *Expert Rev Vaccines* 2011;10(3):307–22.
- [27] Ahl J, Littorin N, Forsgren A, Odenholt I, Resman F, Riesbeck K. High incidence of septic shock caused by *Streptococcus pneumoniae* serotype 3—a retrospective epidemiological study. *BMC Infect Dis* 2013;13:492.
- [28] Andrews NJ, Waite PA, Burbidge P, Pearce E, Roale L, Zancolli M, et al. Serotype-specific effectiveness and correlates of protection for the 13-valent pneumococcal conjugate vaccine: a postlicensure indirect cohort study. *Lancet Infect Dis* 2014;14(9):839–46.
- [29] Richter SS, Heilmann KP, Dohm CL, Riah F, Diekema DJ, Doern GV. Pneumococcal serotypes before and after introduction of conjugate vaccines, United States; 1999–2011. *Emerg Infect Dis* [Internet] July 2013;19(7):on doi: 10.3201/eid1907.121830.
- [30] Yeh SH, Gurtman A, Hurlley DC, Block SL, Schwartz RH, Patterson S, et al. Immunogenicity and safety of 13-valent pneumococcal conjugate vaccine in infants and toddlers. *Pediatrics* 2010;126(3):e493–505.
- [31] Nunes MC, Madhi SA. Review on the immunogenicity and safety of PCV-13 in infants and toddlers. *Expert Rev Vaccines* 2011;10(7):951–80.
- [32] Prymula R, Peeters P, Chrobok V, Kriz P, Novakova E, Kaliskova E, et al. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both *Streptococcus pneumoniae* and non-typable *Haemophilus influenzae*: a randomised double-blind efficacy study. *Lancet* 2006;367(9512):740–8.
- [33] Selva L, Ciruela P, Esteva C, de Sevilla MF, Codina G, Hernandez S, et al. Serotype 3 is a common serotype causing invasive pneumococcal disease in

- children less than 5 years old, as identified by real-time PCR. *Eur J Clin Microbiol Infect Dis* 2012;31(7):1487–95.
- [34] Weinberger DM, Harboe ZB, Sanders EA, Ndiritu M, Klugman KP, Ruckinger S, et al. Association of serotype with risk of death due to pneumococcal pneumonia: a meta-analysis. *Clin Infect Dis* 2010;51(6):692–9.
- [35] Wood Jr WB, Smith MR. The inhibition of surface phagocytosis by the capsular slime layer of pneumococcus type III. *J Exp Med* 1949;90(1):85–96.
- [36] Kalin M. Pneumococcal serotypes and their clinical relevance. *Thorax* 1998;53(3):159–62.
- [37] Who Expert Committee on Biological Standardization. World Health Organ Tech Rep Ser 2005;927:1–154
- [38] Robbins JB, Austrian R, Lee CJ, Rastogi SC, Schiffman G, Henrichsen J, et al. Considerations for formulating the second-generation pneumococcal capsular polysaccharide vaccine with emphasis on the cross-reactive types within groups. *J Infect Dis* 1983;148(6):1136–59.
- [39] Jugenburg M, Haddock G, Freedman MH, Ford-Jones L, Ein SH. The morbidity and mortality of pediatric splenectomy: does prophylaxis make a difference? *J Pediatr Surg* 1999;34(7):1064–7.

# Streptococcus pneumoniae colonization in pneumococcal vaccine-naïve human immunodeficiency virus-exposed infected and -uninfected South African children

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## Abstract

Pneumococcal nasopharyngeal colonization is a pre-requisite for pneumococcal disease; the risk for pneumococcal disease is high in children born to women living with human immunodeficiency virus (HIV). We investigated pneumococcal colonization, serotype distribution and antibiotic susceptibility of *Streptococcus pneumoniae* isolates carried by perinatal HIV-infected and HIV-exposed-uninfected (HEU) children.

Serial nasopharyngeal swabs were collected from 331 HIV-infected and 491 HEU children, at up to 6 scheduled timepoints, between median ages of 25 to 181 weeks. Pneumococcus was identified by culture; serotyping and antibiotic susceptibility testing were done by conventional methods. No pneumococcal vaccine was given.

HIV-infected children were less likely to be colonized with 7-valent pneumococcal conjugate vaccine 7 serotypes than HEU at a median of 25 weeks of age (23% vs 36%;  $P < .001$ ); however, no differences in colonization between the 2 groups were observed at subsequent study-visits. Over the 36-months study-period pneumococcal colonization increased in both HIV-infected (from 45% to 77%) and HEU (from 57% to 61%) children. Over the study-period, pneumococcal isolates non-susceptible to cotrimoxazole decreased from 92% to 57% and had a similar trend to penicillin (from 65% to 42%) in HIV-infected children. Similarly, pneumococcal nonsusceptible to cotrimoxazole decreased from 93% to 57% and to penicillin from 69% to 37% in HEU children.

Vaccine serotype colonization was common in this population and similar rates were observed in HIV-infected and HEU children. The prevalence of pneumococcal isolates non-susceptible to cotrimoxazole and penicillin decreased with age.

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SAM has received honoraria for serving on speaker bureaus for GSK and Pfizer and has also been on advisory boards related to pneumococcal vaccines for GSK, Pfizer, Novartis and MERCK. MCN has received honoraria from Pfizer and travel support from Sanofi-Pasteur.

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**Abbreviations:** GEE = generalized estimating equation, ART = antiretroviral therapy, HEU = HIV-exposed-uninfected, HIV = human immunodeficiency virus, NPS = nasopharyngeal swabs, PCR = polymerase chain reaction, PCV = pneumococcal conjugate vaccine.

**Keywords:** carriage, colonization, HIV, *Streptococcus pneumoniae*

## 1. Introduction

*Streptococcus pneumoniae* (pneumococcus) is a leading cause of childhood morbidity and mortality worldwide.<sup>[1]</sup> Pneumococcal colonization of the human nasopharynx begins in the first months of life.<sup>[2,3]</sup> Although typically asymptomatic, nasopharyngeal carriage is considered a prerequisite for disease.<sup>[4]</sup> Children born to mothers with human immunodeficiency virus (HIV) infection experience high rates of respiratory illness and invasive pneumococcal disease (IPD).<sup>[5]</sup> Although this is partly due to HIV-infection, children exposed to HIV in utero but not infected with it (HIV-exposed uninfected [HEU]) also have higher risk of IPD mortality and hospitalization than HIV-unexposed children.<sup>[6–8]</sup> Nonetheless, studies consistently report higher morbidity in HIV-infected than HEU children.<sup>[6,9]</sup>

There are conflicting reports on whether the increase in IPD in HIV-infected children is related to higher rates of nasopharyngeal colonization.<sup>[10,11]</sup> We have shown no difference in pneumococcal acquisition in vaccine-naïve children on the basis of in utero HIV exposure.<sup>[12]</sup> In that study; however, all children were HEU; therefore, it is important to compare pneumococcal colonization between HIV-infected and HEU children.

Since 2000, the World Health Organization has recommended trimethoprim-sulfamethoxazole (cotrimoxazole) prophylaxis for immunosuppressed adults and children born to women living with HIV starting at 4 to 6 weeks of age and continued until HIV infection can be excluded.<sup>[13]</sup> Implementing widespread and prolonged use of cotrimoxazole prophylaxis could; however, be associated with drug resistance in common colonizing potentially pathogenic organisms.

In this longitudinal study in South Africa, we evaluated pneumococcal colonization prevalence during the first 3 years of life in children born to HIV-infected mothers, as well as antibiotic susceptibility to the main antimicrobials.

## 2. Materials and methods

### 2.1. Study participants, sample collection, and processing

This study addressed a tertiary objective of IMPAACT P1041 (ClinicalTrials.gov NCT00801119), which was a Phase II/III, randomized, double-blind, placebo-controlled trial of HEU and HIV-infected children in whom isoniazid prophylaxis against tuberculosis was evaluated.<sup>[14]</sup> Participants enrolled at Chris Hani Baragwanath Academic Hospital, Johannesburg, South Africa were asked to participate in the pneumococcal colonization sub-study. Infants born to women living with HIV were identified through programs for the prevention of mother-to-child transmission of HIV. Participants were enrolled between December 2004 and June 2008, before the introduction of pneumococcal conjugate vaccine (PCV) into the South African public immunization program. All children in this study were naïve for pneumococcal vaccines. See supplemental text, <http://links.lww.com/MD/D878> for detailed study inclusion and exclusion criteria. When the trial was prematurely stopped for

futility, HEU participants had completed more visits than HIV-infected participants.

The HIV-infection status of the infants was determined by HIV-1 DNA polymerase chain reaction (PCR) testing. Participants were randomly assigned to receive daily isoniazid or placebo. Additionally, open-label daily cotrimoxazole chemoprophylaxis was provided to all HIV-infected children until at least 12-months of age with continuation thereafter if World Health Organization guidelines for continuation were met, and to HEU until their HIV-negative status was confirmed by a repeat HIV-1 DNA PCR and if no longer at risk for acquiring HIV from breastfeeding. Most HIV-infected children began combination antiretroviral therapy (ART) at or after enrolment.

Participants were enrolled at 3 to 4 month of age (Visit-0) and nasopharyngeal swabs (NPS) were collected on 6 occasions spanning a median of 156 weeks; Visit-1 through Visit-6, which occurred at mean ages of 25, 37, 49, 85, 133, and 181 weeks. Dacron-tipped swabs on a flexible aluminium shaft (Wiltshire, England) were inserted gently through a nostril and then inoculated into skim milk tryptone-glucose-glycerol transport media and stored at  $-70^{\circ}\text{C}$  until processing as described previously.<sup>[15]</sup> Standard microbiologic tests were used for culture and identification of *S pneumoniae*.<sup>[15]</sup> A sweep of the growth was used for serotyping. If more than 1 serotype was identified, the individual colonies representative of each morphology were sub-cultured and serotyped. Serotyping was performed with the Quellung method (Statens Serum Institute, Copenhagen, Denmark).

*S pneumoniae* isolates were tested by disc diffusion susceptibility to clindamycin, erythromycin, penicillin, tetracycline, chloramphenicol, and rifampicin (Mast Diagnostics, Merseyside, United Kingdom), and minimal inhibitory concentrations were determined by Etest (AB Biodisk, Solna, Sweden) for cotrimoxazole and ceftriaxone. The results were interpreted according to Clinical and Laboratory Standards Institute guidelines and breakpoint of penicillin non-susceptibility was accordingly meningitis criteria.<sup>[16]</sup>

### 2.2. Statistical analysis

Serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were categorized as 7-valent PCV (PCV7) serotypes and these plus 1, 3, 5, 6A, 7F, 19A were categorized as PCV13-serotypes. To compare characteristics between HIV-infected and HEU participants, Wilcoxon test was used for quantitative variables and Chi-square test or in the case of small cell counts Fisher exact test for binary variables. Separately within HIV status groups, colonization levels were compared by randomized treatment (isoniazid vs placebo) at each study-visit before combining treatment groups. The Kaplan–Meier method was used to calculate the cumulative prevalence by visit, which was defined as the probability of pneumococcal colonization up to and including that visit, censoring at last follow-up if the participant had discontinued the study before the visit. Cumulative prevalence was estimated

with any pneumococci and grouped into PCV7, PCV13, nonvaccine serotypes, not-typeable, and for 6A and 19A stratified by HIV status, and log-rank tests were used to compare HIV-infected and HEU children.

A generalized estimating equation (GEE) approach was used to fit logistic regression models for prevalence of pneumococci at all visits using an exchangeable working correlation structure. Adjusted models included covariates for current (time-updated) cotrimoxazole use, sex, being breastfed at study entry, visit as a categorical variable, and for HIV-infected children, current (time-updated) ART use. A GEE approach was also used to fit ordinal logistic models for antibiotic resistance outcomes using an independent working correlation structure using data from all visits. For both logistic and ordinal logistic models, empirical standard errors were used for confidence intervals and score tests. All *P*-values are 2-sided at the nominal 5% significance level.

### 2.3. Ethics

IMPACT P1041 was approved by the institutional review boards of the participating sites including the Medicines Control Council, Pretoria, South Africa, and the Division of AIDS at National Institute of Allergy and Infectious Diseases, Bethesda, MD, USA; and was registered at ClinicalTrials.gov (NCT00080119). Written informed consent for participation was obtained from legal guardians before any study procedure and from participants in Johannesburg enrolled into this sub-study.

## 3. Results

Overall, of 879 children enrolled in the P1041 study in Johannesburg, 822 (94%) children had NPS results and thus contributed data to this sub-study, including 331 HIV-infected and 491 HEU children. Overall, 405 (49%) were randomized to isoniazid and 417 (51%) to placebo, Table 1. The baseline characteristics were generally similar between both groups although fewer HIV-infected children were male (42% vs 51%, *P*=.01) and a higher proportion were breastfed at enrolment (12% vs 7%, *P*=.02). The mean age at the time of NPS collection for each visit did not differ between groups. The proportion of children receiving cotrimoxazole prophylaxis was, expectedly, higher in the HIV-infected group at all visits. ART was being taken by 68% of HIV-infected children at Visit-1 increasing to 92% by Visit-6, Table 1.

### 3.1. Pneumococcal colonization

HIV-infected children contributed 1085 NPS and HEU contributed 2136 NPS. The percentage of children with pneumococcal colonization was similar between the isoniazid and placebo treatment at all study-visits in both groups; except at Visit-6, based on results from 26 participants, where a lower percentage of HIV-infected children in the isoniazid group were colonized with non-PCV13 serotypes (7%, 1 of 15) compared to children in the placebo group (55%, 6 of 11; *P*=.02).

Overall pneumococcal colonization prevalence was lower at Visit-1 in HIV-infected than HEU (45% vs 57%, *P*=.001) children. This difference was due to lower colonization prevalence by PCV7-serotypes in HIV-infected children (23%) than in HEU (36%), Figures 1 and 2.

Because of a significant interaction effect of HIV status and study-visit in PCV7-serotype colonization, PCV13-serotypes,

and any pneumococci in models adjusted for sex, cotrimoxazole use, and being breastfed at study entry (all *P* ≤ .02), separate models were developed by HIV status and are shown in Table 2. Unadjusted and adjusted logistic regression models showed that being on cotrimoxazole prophylaxis was not associated with PCV7- or PCV13-serotype colonization in both HIV status groups. Prevalence of colonization with any pneumococci was statistically significantly lower while participants were on cotrimoxazole only in the unadjusted model, Table 2. Among HIV-infected children, pneumococcal colonization prevalence generally increased with study-visit for PCV7-serotypes, PCV13-serotypes and any pneumococci in unadjusted and adjusted models (all *P* < .001). ART use was associated with lower prevalence of PCV7-serotype colonization in the adjusted model (*P*=.04), but not for PCV13-serotypes or any pneumococci. Among HEU children, pneumococcal colonization differed by study-visit (significantly in both unadjusted and adjusted models for each pneumococcus grouping, all *P* ≤ .05), but increasing initially and then either plateauing or decreasing, Table 2. Randomization treatment did not predict colonization of PCV7, PCV13, or any pneumococci serotypes or substantially change the associations of other characteristics with prevalence (not shown).

During the study-period, HEU children had PCV7-serotypes cultured at earlier time-points than HIV-infected (*P*=.008), Figure 2 and Supplemental Table 1, <http://links.lww.com/MD/D878>. This was largely driven by the higher prevalence of PCV7-serotypes in HEU at the Visit-1. The estimated cumulative prevalence of colonization with grouped PCV13-serotypes, any PCV13-serotype not in PCV7 or any pneumococci were, however, similar between HIV-infected and HEU children, Figure 2 and Supplemental Table 1, <http://links.lww.com/MD/D878>. We estimated that all HIV-infected children and 96% of HEU children were colonized with at least 1 pneumococcus serotype during the study-period, while 81% each, had at least 1 PCV7-serotype, and 93% and 90%, respectively, had at least 1 PCV13-serotype, Supplemental Table 1, <http://links.lww.com/MD/D878>.

### 3.2. Antibiotic susceptibility

Of samples positive for pneumococcus (629 of 1085 in HIV-infected and 1435 of 2316 in HEU children), antibiotic susceptibility testing was done for cotrimoxazole in 89%, penicillin in 51%, erythromycin in 77%, tetracycline in 87%, clindamycin in 78%, chloramphenicol in 93%, rifampicin in 92% and ceftriaxone in 92%.

The prevalence of isolates non-susceptible (intermediate or resistant) to cotrimoxazole at Visit-1, when >98% of colonized children were receiving cotrimoxazole, was very high (HIV-infected 92% and HEU 93%). In contrast, at Visit-6 when only 1 (0.7%) HEU and 1 (5%) HIV-infected child was on cotrimoxazole, the prevalence of nonsusceptibility had reduced to 57% in both groups, Table 3. Pneumococcus isolated during visits when HIV-infected children were receiving cotrimoxazole were more likely to be nonsusceptible to cotrimoxazole than when children were not receiving cotrimoxazole; however, the odds ratio was attenuated and statistical significance disappeared after adjusting for sex and study-visit, Table 3. Pneumococcus isolated during visits where HEU children received cotrimoxazole showed a similar pattern in unadjusted analysis, with odds ratios attenuated and non-significant after adjusting for sex and visit.

**Table 1**  
Demographic and clinical characteristics of study participants by HIV status.

| Characteristic                                 | Total<br>N=822      | HIV-infected N=331         | HIV-uninfected N=491 | P-value |
|--|---------------------|----------------------------|----------------------|---------|
| Age in weeks at study visit, N, median (range) |                     |                            |                      |         |
| Visit-1/study wk 12                            | 797, 25 (22, 26)    | 310, 25 (22, 36)           | 487, 25 (22, 34)     | .93     |
| Visit-2/study wk 24                            | 748, 37 (34, 37)    | 283, 37 (35, 45)           | 465, 37 (34, 47)     | .83     |
| Visit-3/study wk 36                            | 717, 49 (46, 62)    | 267, 49 (46, 62)           | 450, 49 (47, 57)     | .45     |
| Visit-4/study wk 72                            | 519, 85 (82, 106)   | 157, 85 (82, 93)           | 362, 85 (83, 106)    | .31     |
| Visit-5/study wk 120                           | 350, 133 (129, 167) | 42, 134 (130, 140)         | 308, 133 (129, 167)  | .79     |
| Visit-6/study wk 168                           | 270, 181 (178, 199) | 26, 181 (180, 199)         | 244, 181 (178, 196)  | .61     |
| Treatment group, N (%)                         |                     |                            |                      |         |
| Isoniazid                                      | 405 (49%)           | 164 (50%)                  | 241 (49%)            | .90     |
| Placebo  | 417 (51%)           | 167 (50%)                  | 250 (51%)            |         |
| Receiving cotrimoxazole, N (%)*                |                     |                            |                      |         |
| Visit-1  | 785 (98%)           | 299 (96%)                  | 486 (>99%)           | <.001   |
| Visit-2  | 731 (98%)           | 270 (95%)                  | 461 (99%)            | .002    |
| Visit-3  | 594 (83%)           | 244 (91%)                  | 350 (78%)            | <.001   |
| Visit-4  | 75 (14%)            | 73 (46%)                   | 2 (0.6%)             | <.001   |
| Visit-5  | 11 (3%)             | 9 (21%)                    | 2 (0.6%)             | <.001   |
| Visit-6  | 3 (1%)              | 2 (8%)                     | 1 (0.4%)             | .03     |
| Male, N (%)                                    | 389 (47%)           | 139 (42%)                  | 250 (51%)            | .01     |
| Race/ethnicity, N (%)                          |                     |                            |                      |         |
| Black  | 820 (99.8%)         | 330 (99.7%)                | 490 (99.8%)          | 1.00    |
| Mixed  | 2 (0.2%)            | 1 (0.3%)                   | 1 (0.2%)             |         |
| Housing type, N (%)                            |                     |                            |                      |         |
| Formal (brick) house                           | 569 (69%)           | 230 (69%)                  | 339 (69%)            | .89     |
| Informal (shack/wooden)                        | 253 (31%)           | 101 (31%)                  | 152 (31%)            |         |
| Number living in home, median (IQR)            | 5 (3, 6)            | 5 (3, 6)                   | 5 (3, 7)             | .50     |
| Breastfed at study baseline (%)                | 73 (9%)             | 39 (12%)                   | 34 (7%)              | .02     |
| CDC HIV classification, N (%)                  |                     |                            |                      |         |
| A  |                     | 46 (14%)                   | n/a                  |         |
| B  |                     | 15 (5%)                    | n/a                  |         |
| C  |                     | 2 (1%)                     | n/a                  |         |
| N  |                     | 268 (81%)                  | n/a                  |         |
| Absolute CD4+ cell count, Median (IQR)         |                     | 1826 (1277, 2400)          | n/a                  |         |
| Percent CD4+ cell, Median (IQR)                |                     | 30.10 (24.6, 36.6)         | n/a                  |         |
| HIV-1 RNA (copies/ml), Median (IQR)            |                     | 387,000 (11,000, ≥750,000) | n/a                  |         |
| On ART, N (%)                                  |                     |                            |                      |         |
| Visit-1  |                     | 211 (68%)                  | n/a                  |         |
| Visit-2  |                     | 238 (84%)                  | n/a                  |         |
| Visit-3  |                     | 231 (87%)                  | n/a                  |         |
| Visit-4  |                     | 143 (91%)                  | n/a                  |         |
| Visit-5  |                     | 39 (93%)                   | n/a                  |         |
| Visit-6  |                     | 24 (92%)                   | n/a                  |         |

ART = combination antiretroviral therapy defined as 3 or more antiretroviral drugs from 2 or more classes, IQR = interquartile range, SD = standard deviation.

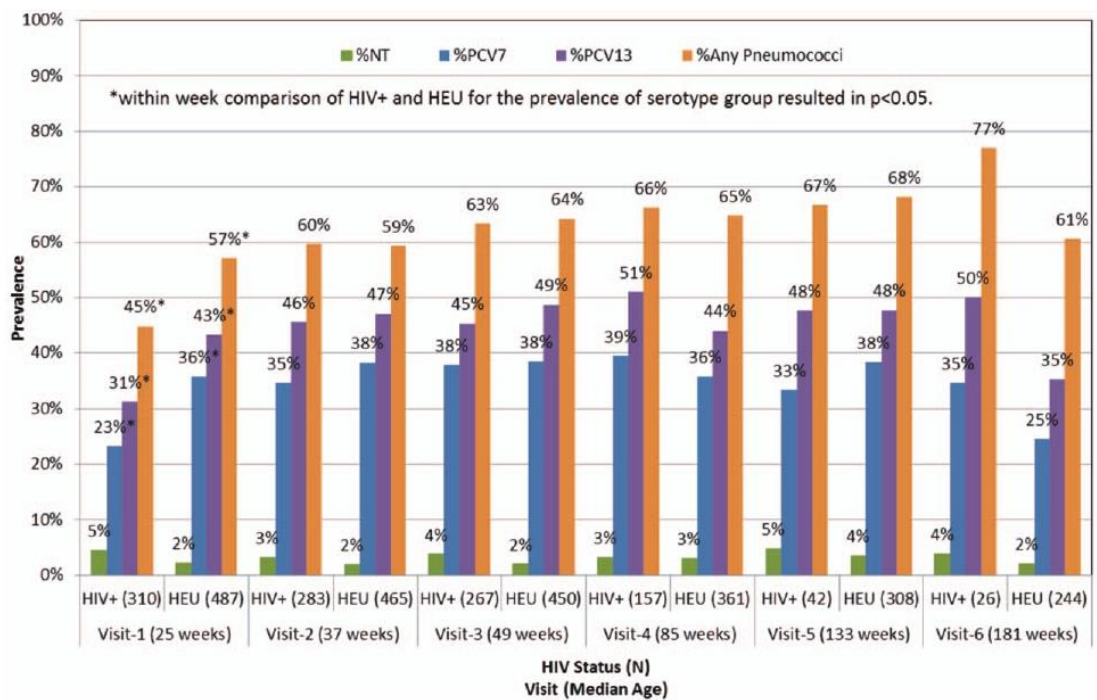
\* For the denominator see age in weeks at study-visit.

The prevalence of isolates non-susceptible to penicillin also generally decreased at later study-visits. Among HIV-infected children, prevalence of penicillin nonsusceptibility was not associated with cotrimoxazole use, and while it decreased with study-visit (65% at Visit-1 to 42% at Visit-6), it was not statistically significant in unadjusted or adjusted models, Table 3. Pneumococcus isolated during visits when HEU children were receiving cotrimoxazole were more frequently nonsusceptible to penicillin (64%) than when not receiving cotrimoxazole (55%,  $P = .03$ ) in unadjusted analysis, although when adjusting for sex and study-visit statistical significance was lost, Table 3.

The prevalence of isolates non-susceptible to erythromycin did not differ significantly by visit in HIV-infected or HEU children but varied between 15% and 23% for HIV-infected children and 14% to 25% for HEU children, Table 3. There was no difference

in the prevalence of non-susceptibility to erythromycin among HIV-infected and HEU children, by receipt of cotrimoxazole.

Nonsusceptibility to tetracycline was identified in 31% of all isolates. HIV-infected children when receiving cotrimoxazole were more likely to have isolates less resistant to tetracycline (unadjusted,  $P = .03$  and adjusted for sex and visit,  $P = .02$ ), Supplemental Table 2, <http://links.lww.com/MD/D878>. Nonsusceptibility to clindamycin was observed in 5% of all isolates. The use of cotrimoxazole had no impact on the prevalence of nonsusceptible isolates to clindamycin in both groups, but this analysis may be underpowered, Supplemental Table 2, <http://links.lww.com/MD/D878>. For the other antibiotics tested, of all isolates <7%, <1%, and 1% were nonsusceptible to chloramphenicol, ceftriaxone, and rifampicin, respectively. No differences in prevalence of nonsusceptible isolates to these antibiotics



**Figure 1.** Prevalence of *Streptococcus pneumoniae* colonization. Number in parenthesis reflect the number of children assessed at each visit. \* $P < .05$  for within week comparison of HIV-infected and HEU children prevalence of serotype group. NT = nontypeable isolates, PCV7 = serotypes included in the 7-valent pneumococcal conjugate vaccine (ie, 4, 6B, 9V, 14, 18C, 19F, 23F), PCV13 = serotypes included in the 13-valent pneumococcal conjugate vaccine (ie, PCV7-serotypes plus 1, 3, 5, 7F).

were detected by cotrimoxazole usage and study-visit (data not shown).

#### 4. Discussion

In the present study PCV-naïve HIV-infected and HEU children had high prevalence of pneumococcal colonization from 25-weeks to 3.5-years of age. While we observed some variation by study-visit, we detected similar prevalence of overall colonization and vaccine serotype colonization in both study groups. In previous studies we have shown that PCV-naïve HEU children were colonized at a similar frequency and by the same pneumococcal serotypes as HIV-unexposed children,<sup>[12]</sup> and we demonstrated that PCV7-immunized HIV-infected and HEU children had similar prevalence of PCV7-serotype colonization to HIV-unexposed children.<sup>[17]</sup> A longitudinal study from Zambia reported; however, that infants born to HIV-infected mothers were at increased risk of pneumococcal colonization compared to HIV-unexposed infants.<sup>[10,18]</sup>

After global implementation of programs for prevention of mother-to-child transmission of HIV, most children born to women living with HIV are now HEU. Nevertheless, these HEU children are at higher risk of morbidity and mortality than children born to HIV-uninfected mothers.<sup>[18,19,20]</sup> Although children with HIV experience higher rates of IPD and mortality, our study did not demonstrate that this group had differential nasal pneumococcal colonization from that of HEU children; but they might be at increased risk of acquiring invasive serotypes

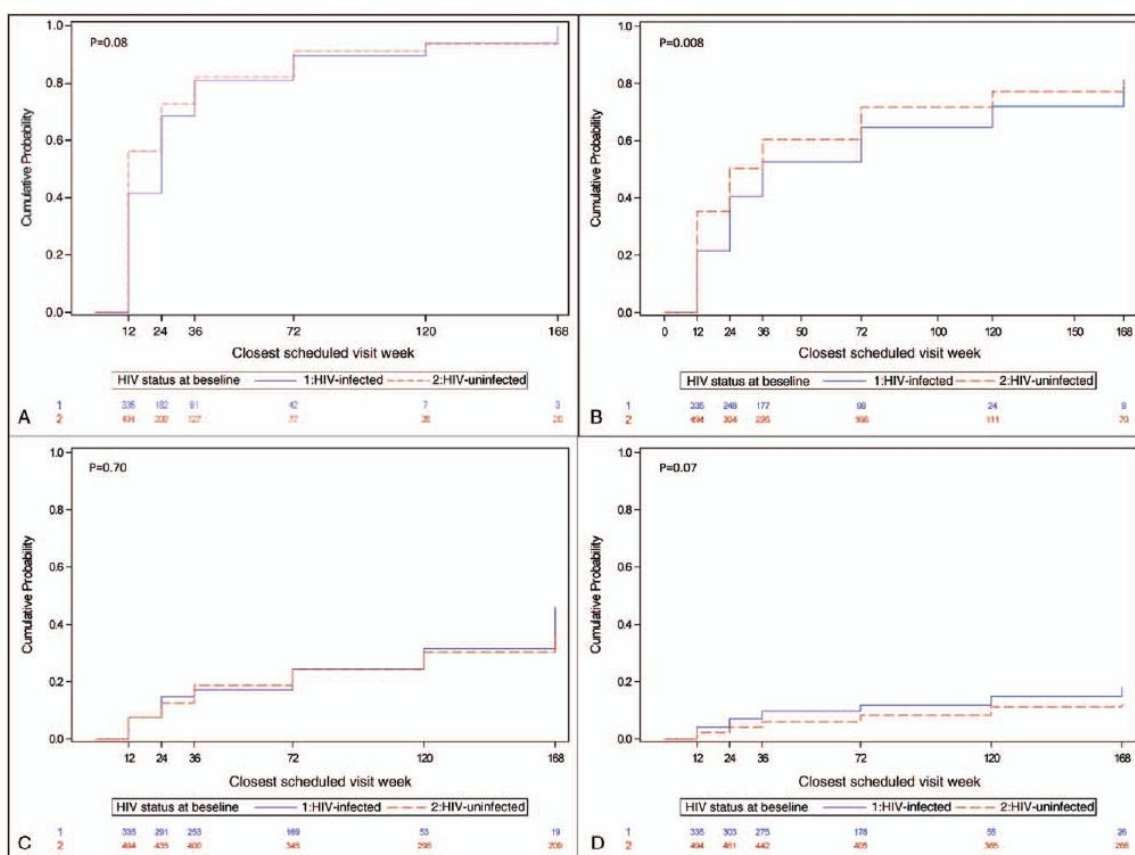
that are very seldom carried in the nasopharynx.<sup>[9]</sup> On the other hand, antibody functional assays on the context of PCV have shown that HIV-exposed children require higher antibody concentrations for effectiveness against certain pneumococcal serotypes.<sup>[21]</sup>

PCV was not used in South Africa during this study. Unsurprisingly, a high colonization prevalence of PCV-serotypes was detected in these children. Since 2009, PCV7, and from 2011, PCV13, are in the national immunization program in South Africa and a gradual reduction in PCV-serotype colonization at the population level has been documented in HIV-infected and uninfected children, most of the latter being HIV-unexposed.<sup>[22]</sup>

Cotrimoxazole is recommended in patients with documented or suspected HIV infection to prevent opportunistic bacterial infections including *Pneumocystis jirovecii* pneumonia.<sup>[23]</sup> A significant correlation has been reported between regional consumption of cotrimoxazole and pneumococci resistance in the following year.<sup>[24,25]</sup> Exposure to sulfonamides is a main factor driving emergence of cotrimoxazole resistant pneumococcus and might induce cross-resistance to other antibiotics.<sup>[26]</sup> While there are concerns that high prevalence of pathogens resistant to cotrimoxazole will reduce the benefits of this intervention, the efficacy of this prophylaxis has not been affected by the background prevalence of cotrimoxazole resistance and appears similar in settings with high and low background prevalence of cotrimoxazole resistance.<sup>[13,27,28]</sup>

In our study cotrimoxazole and penicillin resistance was extremely common, by contrast non-susceptibility to clindamycin





**Figure 2.** Kaplan–Meier estimates of cumulative proportion with Pneumococcal serotype grouping. (A) Kaplan–Meier estimate of cumulative probability of any pneumococci; (B) Kaplan–Meier estimate of cumulative probability of any PCV7-serotypes; (C) Kaplan–Meier estimate of cumulative probability of PCV13-serotypes not included in PCV7; (D) Kaplan–Meier estimate of cumulative probability of nontypeable pneumococcus. PCV = pneumococcal conjugate vaccine.

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cin, ceftriaxone, chloramphenicol, and rifampicin was uncommon. Cotrimoxazole exposure was not associated with cross-resistance to other classes of antibiotics, and resistance level to tetracycline was slightly lower in isolates exposed to cotrimoxazole. A study in South Africa on IPD isolates collected from 2003 to 2007 reported that patients on cotrimoxazole prophylaxis were more likely to have a pneumococcal isolates nonsusceptible to cotrimoxazole (84% vs 54%), penicillin (49% vs 30%), and rifampicin (11% vs 3%) and having multidrug resistance (27% vs 16%).<sup>[29]</sup> In contrast to our study a report from the Zambia among HIV-exposed infants (14% were HIV-infected), found that cotrimoxazole prophylaxis increased pneumococcal resistance to clindamycin, yet had also no effect on resistance to penicillin, tetracycline, erythromycin, and chloramphenicol.<sup>[18]</sup> Although limited by inclusion of few studies (8 of high quality) a systematic-review on whether cotrimoxazole prophylaxis in HIV-infected and/or exposed individuals increased bacterial resistance to other antibiotics suggested that cotrimoxazole usage protected against developing resistance to other antibiotic classes. The authors noted, however that more carefully designed studies are needed to answer the question conclusively.<sup>[30]</sup>

Our study had limited power to look at differences in colonization by individual serotypes but was better powered to detect PCV-serotypes as a group. We included in our analysis the PCV7-serotypes as these have been described as the “paediatric-serotypes” and the most dominant colonizers in pre-vaccine era.<sup>[31]</sup> Other limitations include that we used cumulative prevalence to describe the level of pneumococcal colonization of over a period of time. Cumulative prevalence is not a well-defined quantity and combines prevalence at single point in time and incidence. Because this study was conducted in children starting at approximately 6 months of age, cumulative prevalence in this context may, however approximate cumulative incidence. Nevertheless, because pneumococcus can be cleared between visits, the estimates presented may underestimate the true values. Another limitation is that only 26 HIV-infected children attended the last study-visit.

In this study up to 3.5-years of age, HIV-infected and HEU children had similarly high pneumococcus colonization. Pneumococcal colonization and serotype distribution were also not influenced by cotrimoxazole exposure, suggesting that the need for pneumococcal vaccines is unchanged with the use cotrimoxazole prophylaxis.

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**Table 2**  
Modeling nasal pneumococcal colonization by serotype group by HIV status.\*

| HIV-infected covariate | PCV7 serotype |                |                                     | PCV13 serotype |                          |                | Any pneumococci                     |       |                          |                |                                     |       |
|------------------------|---------------|----------------|-------------------------------------|----------------|--------------------------|----------------|-------------------------------------|-------|--------------------------|----------------|-------------------------------------|-------|
|                        | N             | % <sup>†</sup> | Adjusted <sup>‡</sup><br>OR (95%CI) | P              | Unadjusted<br>OR (95%CI) | % <sup>†</sup> | Adjusted <sup>‡</sup><br>OR (95%CI) | P     | Unadjusted<br>OR (95%CI) | % <sup>†</sup> | Adjusted <sup>‡</sup><br>OR (95%CI) | P     |
| Taking COT             |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Yes                    | 897           | 33%            | 1.35 (0.89, 2.04)                   | .17            | 0.85 (0.60, 1.19)        | 42%            | 1.42 (0.94, 2.14)                   | .10   | 0.65 (0.46, 0.92)        | 57%            | 1.09 (0.71, 1.68)                   | .69   |
| No                     | 188           | 34%            | ref.                                |                | ref.                     | 44%            | ref.                                |       | ref.                     | 65%            | ref.                                |       |
| Sex                    |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Female                 | 621           | 34%            | 1.17 (0.85, 1.61)                   | .32            | 1.22 (0.91, 1.63)        | 44%            | 1.24 (0.92, 1.68)                   | .16   | 1.26 (0.95, 1.68)        | 60%            | 1.26 (0.94, 1.70)                   | .12   |
| Male                   | 464           | 31%            | ref.                                |                | ref.                     | 40%            | ref.                                |       | ref.                     | 55%            | ref.                                |       |
| ART                    |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Yes                    | 886           | 32%            | 0.65 (0.44, 0.95)                   | .04            | 1.04 (0.74, 1.45)        | 42%            | 0.77 (0.54, 1.10)                   | .17   | 1.18 (0.82, 1.71)        | 58%            | 0.86 (0.58, 1.27)                   | .45   |
| No                     | 199           | 36%            | ref.                                |                | ref.                     | 42%            | ref.                                |       | ref.                     | 56%            | ref.                                |       |
| Breastfed <sup>§</sup> |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Yes                    | 134           | 30%            | 0.80 (0.50, 1.27)                   | .33            | 0.86 (0.58, 1.27)        | 40%            | 0.81 (0.54, 1.20)                   | .29   | 0.99 (0.65, 1.49)        | 57%            | 0.94 (0.62, 1.44)                   | .78   |
| No                     | 951           | 33%            | ref.                                |                | ref.                     | 43%            | ref.                                |       | ref.                     | 58%            | ref.                                |       |
| Study Visit            |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Visit-1                | 310           | 23%            | ref.                                | <.001          | ref.                     | 31%            | ref.                                | <.001 | ref.                     | 45%            | ref.                                | <.001 |
| Visit-2                | 283           | 35%            | 1.71 (1.26, 2.32)                   |                | 1.79 (1.34, 2.41)        | 46%            | 1.89 (1.40, 2.55)                   |       | 1.78 (1.33, 2.38)        | 60%            | 1.83 (1.36, 2.47)                   |       |
| Visit-3                | 267           | 38%            | 1.99 (1.46, 2.72)                   |                | 1.80 (1.34, 2.41)        | 45%            | 1.93 (1.42, 2.62)                   |       | 2.10 (1.57, 2.81)        | 63%            | 2.18 (1.62, 2.93)                   |       |
| Visit-4                | 157           | 39%            | 2.13 (1.40, 3.25)                   |                | 2.39 (1.63, 3.51)        | 51%            | 3.07 (1.98, 4.77)                   |       | 2.55 (1.73, 3.77)        | 66%            | 2.79 (1.75, 4.43)                   |       |
| Visit-5                | 42            | 33%            | 1.70 (0.85, 3.40)                   |                | 2.40 (1.18, 4.88)        | 48%            | 2.93 (1.44, 6.00)                   |       | 2.70 (1.39, 5.25)        | 67%            | 3.00 (1.46, 6.15)                   |       |
| Visit-6                | 26            | 35%            | 1.84 (0.79, 4.31)                   |                | 2.82 (1.17, 6.82)        | 50%            | 3.57 (1.40, 9.07)                   |       | 4.81 (1.60, 14.4)        | 77%            | 5.46 (1.63, 18.3)                   |       |
| Taking COT             |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Yes                    | 1302          | 37%            | 1.15 (0.95, 1.38)                   | .15            | 1.14 (0.95, 1.35)        | 46%            | 1.13 (0.76, 1.68)                   | .55   | 0.83 (0.70, 0.98)        | 60%            | 1.23 (0.81, 1.86)                   | .35   |
| No                     | 1014          | 34%            | ref.                                |                | ref.                     | 43%            | ref.                                |       | ref.                     | 64%            | ref.                                |       |
| Sex                    |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Female                 | 1136          | 35%            | 0.94 (0.76, 1.16)                   | .54            | 0.99 (0.80, 1.23)        | 45%            | 0.99 (0.80, 1.22)                   | .93   | 1.00 (0.80, 1.25)        | 62%            | 1.00 (0.80, 1.25)                   | .99   |
| Male                   | 1180          | 37%            | ref.                                |                | ref.                     | 45%            | ref.                                |       | ref.                     | 62%            | ref.                                |       |
| Breastfed <sup>§</sup> |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Yes                    | 166           | 37%            | 1.06 (0.67, 1.67)                   | .80            | 0.90 (0.58, 1.41)        | 42%            | 0.89 (0.57, 1.40)                   | .62   | 0.94 (0.61, 1.43)        | 60%            | 0.92 (0.60, 1.42)                   | .71   |
| No                     | 2150          | 36%            | ref.                                |                | ref.                     | 45%            | ref.                                |       | ref.                     | 62%            | ref.                                |       |
| Study Visit            |               |                |                                     |                |                          |                |                                     |       |                          |                |                                     |       |
| Visit-1                | 487           | 36%            | ref.                                | .002           | ref.                     | 43%            | ref.                                | .01   | ref.                     | 57%            | ref.                                | .05   |
| Visit-2                | 465           | 38%            | 1.11 (0.90, 1.36)                   |                | 1.16 (0.95, 1.41)        | 47%            | 1.16 (0.96, 1.41)                   |       | ref.                     | 59%            | 1.10 (0.90, 1.35)                   |       |
| Visit-3                | 450           | 38%            | 1.12 (0.88, 1.42)                   |                | 1.23 (0.98, 1.53)        | 49%            | 1.26 (0.99, 1.60)                   |       | 1.35 (1.08, 1.69)        | 64%            | 1.41 (1.11, 1.80)                   |       |
| Visit-4                | 362           | 36%            | 1.00 (0.75, 1.33)                   |                | 1.03 (0.78, 1.35)        | 44%            | 1.16 (0.71, 1.89)                   |       | 1.40 (1.07, 1.84)        | 65%            | 1.72 (1.03, 2.86)                   |       |
| Visit-5                | 308           | 38%            | 1.11 (0.84, 1.47)                   |                | 1.05 (0.63, 1.75)        | 48%            | 1.34 (0.82, 2.18)                   |       | 1.62 (1.22, 2.15)        | 68%            | 1.98 (1.16, 3.38)                   |       |
| Visit-6                | 244           | 25%            | 0.58 (0.41, 0.81)                   |                | 0.54 (0.31, 0.95)        | 35%            | 0.79 (0.48, 1.31)                   |       | 1.17 (0.86, 1.58)        | 61%            | 1.43 (0.85, 2.43)                   |       |

CI=confidence interval, COT=cotrimoxazole, N=number of participant visits, OR=odds ratio, P=P-value, ref.=reference group.  
\* Generalized estimating equations used to fit logistic regression models. Adjusted models have current cotrimoxazole usage, sex, ever breastfed evaluated at study entry, visit, and for HIV+ participants, current ART use, as covariates.  
<sup>†</sup> Percentage of participant visits colonized.  
<sup>‡</sup> Adjusted models fit separately for HIV-infected and HIV-uninfected participants containing covariates shown in table.  
<sup>§</sup> Breastfed evaluated at study entry, very few children were breastfed on study.

**Table 3**  
Unadjusted and adjusted models of resistance to cotrimoxazole, penicillin and erythromycin separately by participant's HIV status\*

| HIV-infected covariate | Pneumococci isolated N (%) <sup>†</sup> | Cotrimoxazole (N=570) |       |       |       |                |       |                       |       | Penicillin (N=299) |       |       |                |     |                       |     |
|------------------------|---|-----------------------|-------|-------|-------|----------------|-------|-----------------------|-------|--------------------|-------|-------|----------------|-----|-----------------------|-----|
|                        |   | N                     | S     | I     | R     | Unadjusted     |       | Adjusted <sup>‡</sup> |       | N                  | S     | No-S  | Unadjusted     |     | Adjusted <sup>‡</sup> |     |
|                        |   |                       |       |       |       | OR (95%CI)     | P     | OR (95%CI)            | P     |                    |       |       | OR (95%CI)     | P   | OR (95%CI)            | P   |
| Visit Week             |   |                       |       |       |       |                |       |                       |       |                    |       |       |                |     |                       |     |
| 12                     | 139 (44.8%)                             | 135                   | 8.1%  | 39.3% | 52.6% | ref.           | <.001 | ref.                  | .007  | 46                 | 34.8% | 65.2% | ref.           | .39 | ref.                  | .40 |
| 24                     | 169 (59.7%)                             | 153                   | 7.8%  | 27.5% | 64.7% | 1.5 (1.0, 2.4) |       | 1.6 (1.0, 2.4)        |       | 61                 | 47.5% | 52.5% | 0.6 (0.3, 1.3) |     | 0.6 (0.3, 1.3)        |     |
| 36                     | 169 (59.7%)                             | 157                   | 12.1% | 37.6% | 50.3% | 0.9 (0.6, 1.3) |       | 0.9 (0.6, 1.3)        |       | 104                | 36.5% | 63.5% | 0.9 (0.4, 1.9) |     | 0.9 (0.5, 1.9)        |     |
| 72                     | 104 (66.2%)                             | 86                    | 22.1% | 40.7% | 37.2% | 0.5 (0.3, 0.8) |       | 0.6 (0.3, 1.1)        |       | 56                 | 33.9% | 66.1% | 1.0 (0.5, 2.3) |     | 1.1 (0.4, 2.8)        |     |
| 120                    | 28 (66.7%)                              | 25                    | 36.0% | 36.0% | 28.0% | 0.3 (0.1, 0.6) |       | 0.3 (0.1, 0.8)        |       | 20                 | 45.0% | 55.0% | 0.7 (0.2, 1.9) |     | 0.7 (0.2, 2.6)        |     |
| 168                    | 20 (75.9%)                              | 14                    | 42.9% | 21.4% | 35.7% | 0.3 (0.1, 0.9) |       | 0.3 (0.1, 1.0)        |       | 12                 | 58.3% | 41.7% | 0.4 (0.1, 1.3) |     | 0.4 (0.1, 1.8)        |     |
| Taking COT             |   |                       |       |       |       |                |       |                       |       |                    |       |       |                |     |                       |     |
| Yes                    | 507 (56.5%)                             | 468                   | 10.5% | 34.8% | 54.7% | 2.4 (1.6, 3.7) | <.001 | 1.4 (0.8, 2.5)        | .30   | 228                | 38.6% | 61.4% | 1.2 (0.7, 2.0) | .60 | 1.1 (0.5, 2.5)        | .80 |
| No                     | 122 (64.9%)                             | 102                   | 26.5% | 37.3% | 36.3% | ref.           |       | ref.                  |       | 71                 | 42.3% | 57.7% | ref.           |     | ref.                  |     |
| ART                    |   |                       |       |       |       |                |       |                       |       |                    |       |       |                |     |                       |     |
| Yes                    | 517 (58.4%)                             | 467                   | 13.7% | 35.8% | 50.5% | 0.8 (0.5, 1.3) | .42   | ref.                  |       | 249                | 39.4% | 60.6% | 1.0 (0.5, 2.0) | .94 | ref.                  |     |
| No                     | 112 (56.3%)                             | 103                   | 11.7% | 33.0% | 55.3% | ref.           |       | ref.                  |       | 50                 | 40.0% | 60.0% | ref.           |     | ref.                  |     |
| Sex                    |   |                       |       |       |       |                |       |                       |       |                    |       |       |                |     |                       |     |
| Male                   | 255 (55.0%)                             | 230                   | 14.3% | 44.3% | 41.3% | 0.6 (0.4, 0.8) | .001  | 0.5 (0.4, 0.8)        | <.001 | 127                | 38.6% | 61.4% | 1.1 (0.6, 1.8) | .80 | 1.0 (0.6, 1.7)        | .92 |
| Female                 | 374 (60.2%)                             | 340                   | 12.6% | 29.1% | 58.2% | ref.           |       | ref.                  |       | 172                | 40.1% | 59.9% | ref.           |     | ref.                  |     |

| HIV-uninfected covariate | Pneumococci isolated N (%) <sup>†</sup> | Cotrimoxazole (N=1276) |       |       |       |                |       |                       |       | Penicillin (N=755) |       |       |                |      |                       |      |
|--------------------------|---|------------------------|-------|-------|-------|----------------|-------|-----------------------|-------|--------------------|-------|-------|----------------|------|-----------------------|------|
|                          |   | N                      | S     | I     | R     | Unadjusted     |       | Adjusted <sup>‡</sup> |       | N                  | S     | No-S  | Unadjusted     |      | Adjusted <sup>‡</sup> |      |
|                          |   |                        |       |       |       | OR (95%CI)     | P     | OR (95%CI)            | P     |                    |       |       | OR (95%CI)     | P    | OR (95%CI)            | P    |
| Visit Week               |   |                        |       |       |       |                |       |                       |       |                    |       |       |                |      |                       |      |
| 12                       | 278 (57.1%)                             | 273                    | 7.0%  | 17.9% | 75.1% | ref.           | <.001 | ref.                  | <.001 | 156                | 31.4% | 68.6% | ref.           | .002 | ref.                  | .002 |
| 24                       | 276 (59.4%)                             | 256                    | 3.9%  | 16.8% | 79.3% | 1.3 (0.9, 1.9) |       | 1.3 (0.9, 1.9)        |       | 105                | 36.2% | 63.8% | 0.8 (0.5, 1.3) |      | 0.8 (0.5, 1.3)        |      |
| 36                       | 289 (64.2%)                             | 254                    | 18.1% | 22.8% | 59.1% | 0.4 (0.3, 0.6) |       | 0.5 (0.3, 0.7)        |       | 143                | 38.5% | 61.5% | 0.7 (0.5, 1.1) |      | 0.6 (0.4, 1.0)        |      |
| 72                       | 234 (64.6%)                             | 224                    | 31.3% | 37.5% | 31.3% | 0.2 (0.1, 0.2) |       | 0.3 (0.1, 0.5)        |       | 155                | 38.7% | 61.3% | 0.7 (0.5, 1.1) |      | 0.4 (0.1, 0.9)        |      |
| 120                      | 210 (68.2%)                             | 170                    | 27.1% | 31.8% | 41.2% | 0.2 (0.2, 0.3) |       | 0.3 (0.2, 0.7)        |       | 113                | 46.0% | 54.0% | 0.5 (0.3, 0.9) |      | 0.3 (0.1, 0.7)        |      |
| 168                      | 148 (60.7%)                             | 99                     | 43.4% | 23.2% | 33.3% | 0.1 (0.1, 0.2) |       | 0.2 (0.1, 0.4)        |       | 83                 | 62.7% | 37.3% | 0.3 (0.2, 0.5) |      | 0.1 (0.0, 0.4)        |      |
| Taking COT               |   |                        |       |       |       |                |       |                       |       |                    |       |       |                |      |                       |      |
| Yes                      | 784 (60.2%)                             | 731                    | 9.4%  | 17.5% | 73.1% | 4.6 (3.7, 5.8) | <.001 | 1.5 (0.9, 2.6)        | .09   | 375                | 36.0% | 64.0% | 1.5 (1.0, 2.0) | .03  | 0.5 (0.2, 1.2)        | .09  |
| No                       | 651 (64.2%)                             | 545                    | 30.3% | 33.6% | 36.1% | ref.           |       | ref.                  |       | 380                | 45.0% | 55.0% | ref.           |      | ref.                  |      |
| Sex                      |   |                        |       |       |       |                |       |                       |       |                    |       |       |                |      |                       |      |
| Male                     | 731 (61.9%)                             | 646                    | 19.2% | 22.0% | 58.8% | 1.1 (0.9, 1.3) | .56   | 1.0 (0.8, 1.3)        | .89   | 376                | 38.3% | 61.7% | 1.2 (0.9, 1.6) | .24  | 1.2 (0.9, 1.7)        | .25  |
| Female                   | 704 (62.0%)                             | 630                    | 17.5% | 26.8% | 55.7% | ref.           |       | ref.                  |       | 379                | 42.7% | 57.3% | ref.           |      | ref.                  |      |

| HIV-infected covariate | N   | S     | I    | R     | Erythromycin (N=479) |                       |
|------------------------|-----|-------|------|-------|----------------------|-----------------------|
|                        |     |       |      |       | Unadjusted           | Adjusted <sup>‡</sup> |
|                        |     |       |      |       | OR (95%CI)           | P                     |
| Visit Week             |     |       |      |       |                      |                       |
| 12                     | 106 | 84.9% | 2.8% | 12.3% |                      | .56                   |
| 24                     | 131 | 85.5% | 2.3% | 12.2% | ref.                 |                       |
| 36                     | 133 | 77.4% | 5.3% | 17.3% | 1.0 (0.5, 1.9)       |                       |
| 72                     | 75  | 80.0% | 2.7% | 17.3% | 1.6 (0.9, 3.0)       |                       |
| 120                    | 22  | 77.3% | 4.5% | 18.2% | 1.4 (0.6, 3.2)       |                       |
| 168                    | 12  | 83.3% | 8.3% | 8.3%  | 1.6 (0.5, 5.2)       |                       |
| Taking COT             |     |       |      |       |                      |                       |
| Yes                    | 395 | 82.5% | 3.5% | 13.9% |                      | .43                   |
| No                     | 84  | 78.6% | 3.6% | 17.9% | 0.8 (0.4, 1.4)       |                       |
| ART                    |     |       |      |       |                      |                       |
| Yes                    | 389 | 81.2% | 3.6% | 15.2% |                      | .46                   |
| No                     | 90  | 84.4% | 3.3% | 12.2% | 1.3 (0.7, 2.4)       |                       |
| Sex                    |     |       |      |       |                      |                       |
| Male                   | 201 | 80.1% | 4.5% | 15.4% |                      | .45                   |
| Female                 | 278 | 83.1% | 2.9% | 14.0% | 1.2 (0.7, 2.0)       |                       |
| Female                 | 106 | 84.9% | 2.8% | 12.3% | ref.                 |                       |

| HIV-uninfected covariate | N   | S     | I    | R     | Erythromycin (N=1114) |                       |
|--------------------------|-----|-------|------|-------|-----------------------|-----------------------|
|                          |     |       |      |       | Unadjusted            | Adjusted <sup>‡</sup> |
|                          |     |       |      |       | OR (95%CI)            | P                     |
| Visit Week               |     |       |      |       |                       |                       |
| 12                       | 222 | 86.5% | 1.4% | 12.2% | ref.                  | .12                   |
| 24                       | 225 | 83.1% | 4.9% | 12.0% | 1.3 (0.8, 1.9)        |                       |
| 36                       | 243 | 83.5% | 4.1% | 12.3% | 1.2 (0.8, 1.9)        |                       |
| 72                       | 193 | 86.0% | 2.6% | 11.4% | 1.0 (0.6, 1.8)        |                       |
| 120                      | 140 | 75.0% | 4.3% | 20.7% | 2.1 (1.2, 3.7)        |                       |
| 168                      | 91  | 85.7% | 7.7% | 6.6%  | 1.0 (0.5, 2.0)        |                       |
| Taking COT               |     |       |      |       |                       |                       |
| Yes                      | 97  | 86.6% | 7.2% | 6.2%  |                       | .59                   |
| No                       | 637 | 84.1% | 3.6% | 12.2% | 0.9 (0.6, 1.3)        |                       |
| Sex                      |     |       |      |       |                       |                       |
| Male                     | 477 | 82.8% | 4.0% | 13.2% | ref.                  | .77                   |
| Female                   | 555 | 84.0% | 3.6% | 12.4% | 0.9 (0.7, 1.4)        |                       |
| Female                   | 559 | 83.2% | 3.9% | 12.9% | ref.                  |                       |

CI= confidence interval, COT=cotrimoxazole, I=intermediate, N=number of participant visits, No-S=nonsusceptible, P=P-value, R=resistant, ref.= reference group, S=susceptible.

\* Generalized estimating equations used to fit cumulative logit models for HIV-infected and HIV-uninfected groups separately.

‡ Adjusted models have cotrimoxazole usage, sex, and visit as covariates.

† N (%): Number of participant visits with any pneumococci isolated (percentage of total participant visits with results reported).

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## Author contributions

AV, MFC, CM and SAM designed the study. SD, HC, TT, NvN, PVA and SAM acquired the data. MCN, SK, BZ, NvN and PVA analysed the data. All authors participated in the interpretation of the data. All reviewed and revised the manuscript. Marta Nunes orcid: 0000-0003-3788-878X.

## References

- O'Brien KL, Wolfson LJ, Watt JP, et al. Burden of disease caused by *Streptococcus pneumoniae* in children younger than 5 years: global estimates. *Lancet* 2009;374:893–902.
- Tigoi CC, Gatakaa H, Karani A, et al. Rates of acquisition of pneumococcal colonization and transmission probabilities, by serotype, among newborn infants in Kilifi District, Kenya. *Clin Infect Dis* 2012;55:180–8.
- Turner P, Turner C, Jankhot A, et al. A longitudinal study of *Streptococcus pneumoniae* carriage in a cohort of infants and their mothers on the Thailand-Myanmar border. *PLoS One* 2012;7:e38271.
- Bogaert D, De Groot R, Hermans PW. *Streptococcus pneumoniae* colonisation: the key to pneumococcal disease. *Lancet Infect Dis* 2004;4:144–54.
- Nunes MC, von Gottberg A, de Gouveia L, et al. The impact of antiretroviral treatment on the burden of invasive pneumococcal disease in South African children: a time series analysis. *AIDS* 2011;25:453–62.
- von Mollendorf C, von Gottberg A, Tempia S, et al. Increased risk for and mortality from invasive pneumococcal disease in HIV-exposed but uninfected infants aged <1 year in South Africa, 2009–2013. *Clin Infect Dis* 2015;60:1346–56.
- Slogrove A, Reikie B, Naidoo S, et al. HIV-exposed uninfected infants are at increased risk for severe infections in the first year of life. *J Trop Pediatr* 2012;58:505–8.
- McNally LM, Jeena PM, Gajee K, et al. Effect of age, polymicrobial disease, and maternal HIV status on treatment response and cause of severe pneumonia in South African children: a prospective descriptive study. *Lancet* 2007;369:1440–51.
- Cohen C, Moyes J, Tempia S, et al. Epidemiology of acute lower respiratory tract infection in HIV-exposed uninfected infants. *Pediatrics* 2016;137:e20153272.
- Gill CJ, Mwanakasale V, Fox MP, et al. Impact of human immunodeficiency virus infection on *Streptococcus pneumoniae* colonization and seroepidemiology among Zambian women. *J Infect Dis* 2008;197:1000–5.
- Polack FP, Flayhart DC, Zahurak ML, et al. Colonization by *Streptococcus pneumoniae* in human immunodeficiency virus-infected children. *Pediatr Infect Dis J* 2000;19:608–12.
- Nunes MC, Shiri T, van Niekerk N, et al. Acquisition of *Streptococcus pneumoniae* in pneumococcal conjugate vaccine-naïve South African children and their mothers. *Pediatr Infect Dis J* 2013;32:e192–205.
- World Health Organisation, Guidelines on Co-trimoxazole Prophylaxis for HIV Related Infections Among Children, Adolescents and adults: Recommendations for a Public Health Approach, 2006; 2017. [Accessed 15 November 2017]
- Madhi SA, Nachman S, Violari A, et al. Primary isoniazid prophylaxis against tuberculosis in HIV-exposed children. *N Engl J Med* 2011;365:21–31.
- Madhi SA, Adrian P, Kuwanda L, et al. Long-term effect of pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae*—and associated interactions with *Staphylococcus aureus* and *Haemophilus influenzae* colonization—in HIV-Infected and HIV-uninfected children. *J Infect Dis* 2007;196:1662–6.
- CLSI Performance Standards for Antimicrobial Susceptibility Testing: Nineteenth Informational Supplement. CLSI Document M100-S21. Wayne, PA: Clinical and Laboratory Standards Institute; 2011.
- Madhi SA, Izu A, Nunes MC, et al. Longitudinal study on *Streptococcus pneumoniae*, *Haemophilus influenzae* and *Staphylococcus aureus* nasopharyngeal colonization in HIV-infected and -uninfected infants vaccinated with pneumococcal conjugate vaccine. *Vaccine* 2015;33:2662–9.
- Gill CJ, Mwanakasale V, Fox MP, et al. Effect of presumptive co-trimoxazole prophylaxis on pneumococcal colonization rates, seroepidemiology and antibiotic resistance in Zambian infants: a longitudinal cohort study. *Bull World Health Organ* 2008;86:929–38.
- Newell ML, Coovadia H, Cortina-Borja M, et al. Mortality of infected and uninfected infants born to HIV-infected mothers in Africa: a pooled analysis. *Lancet* 2004;364:1236–43.
- Taron-Brocard C, Le Chenadec J, Faye A, et al. Increased risk of serious bacterial infections due to maternal immunosuppression in HIV-exposed uninfected infants in a European country. *Clin Infect Dis* 2014;59:1332–45.
- Madhi SA, Adrian P, Cotton MF, et al. Effect of HIV infection status and anti-retroviral treatment on quantitative and qualitative antibody responses to pneumococcal conjugate vaccine in infants. *J Infect Dis* 2010;202:355–61.
- Nzenze SA, von Gottberg A, Shiri T, et al. Temporal changes in pneumococcal colonization in HIV-infected and HIV-uninfected mother-child pairs following transitioning from 7-valent to 13-valent pneumococcal conjugate vaccine, Soweto, South Africa. *J Infect Dis* 2015;212:1082–92.
- Chintu C, Bhat GJ, Walker AS, et al. Co-trimoxazole as prophylaxis against opportunistic infections in HIV-infected Zambian children (CHAP): a double-blind randomised placebo-controlled trial. *Lancet* 2004;364:1865–71.
- Karpanoja P, Nyberg ST, Bergman M, et al. Connection between trimethoprim-sulfamethoxazole use and resistance in *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Moraxella catarrhalis*. *Antimicrob Agents Chemother* 2008;52:2480–5.
- Sanaei Dashti A, Abdinia B, Karimi A. Nasopharyngeal carrier rate of *Streptococcus pneumoniae* in children: serotype distribution and antimicrobial resistance. *Arch Iran Med* 2012;15:500–3.
- Porat N, Leibovitz E, Dagan R, et al. Molecular typing of *Streptococcus pneumoniae* in northeastern Romania: unique clones of *S. pneumoniae* isolated from children hospitalized for infections and from healthy and human immunodeficiency virus-infected children in the community. *J Infect Dis* 2000;181:966–74.
- Zachariah R, Harries AD, Luo C, et al. Scaling-up co-trimoxazole prophylaxis in HIV-exposed and HIV-infected children in high HIV-prevalence countries. *Lancet Infect Dis* 2007;7:686–93.
- Nunn AJ, Mwaba P, Chintu C, et al. Role of co-trimoxazole prophylaxis in reducing mortality in HIV infected adults being treated for tuberculosis: randomised clinical trial. *BMJ* 2008;337:a257.
- Soeters HM, von Gottberg A, Cohen C, et al. Trimethoprim-sulfamethoxazole prophylaxis and antibiotic nonsusceptibility in invasive pneumococcal disease. *Antimicrob Agents Chemother* 2012;56:1602–5.
- Sibanda EL, Weller IV, Hakim JG, et al. Does trimethoprim-sulfamethoxazole prophylaxis for HIV induce bacterial resistance to other antibiotic classes? Results of a systematic review. *Clin Infect Dis* 2011;52:1184–94.
- Nzenze SA, Shiri T, Nunes MC, et al. Temporal changes in pneumococcal colonization in a rural African community with high HIV prevalence following routine infant pneumococcal immunization. *Pediatr Infect Dis J* 2013;32:1270–8.



# Pneumococcal Conjugate Vaccine Protection against Coronavirus-Associated Pneumonia Hospitalization in Children Living with and without HIV

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**ABSTRACT** In December 2019 a new coronavirus (CoV) emerged as a human pathogen, SARS-CoV-2. There are few data on human coronavirus infections among individuals living with HIV. In this study we probed the role of pneumococcal coinfections with seasonal CoVs among children living with and without HIV hospitalized for pneumonia. We also described the prevalence and clinical manifestations of these infections. A total of 39,836 children who participated in a randomized, double-blind, placebo-controlled clinical trial on the efficacy of a 9-valent pneumococcal conjugate vaccine (PCV9) were followed for lower respiratory tract infection hospitalizations until 2 years of age. Nasopharyngeal aspirates were collected at the time of hospitalization and were screened by PCR for four seasonal CoVs. The frequency of CoV-associated pneumonia was higher in children living with HIV (19.9%) than in those without HIV (7.6%,  $P < 0.001$ ). Serial CoV infections were detected in children living with HIV. The case fatality risk among children with CoV-associated pneumonia was higher in those living with HIV (30.4%) than without HIV (2.9%,  $P = 0.001$ ). C-reactive protein and procalcitonin levels were elevated in 36.8% ( $\geq 40$  mg/liter) and 64.7% ( $\geq 0.5$  ng/ml), respectively, of the fatal cases living with HIV. Among children without HIV, there was a 64.0% (95% CI: 22.9% to 83.2%) lower incidence of CoV-associated pneumonia hospitalizations among PCV9 recipients compared to placebo recipients. These data suggest that *Streptococcus pneumoniae* infections might have a role in the development of pneumonia associated with endemic CoVs, that PCV may prevent pediatric CoV-associated hospitalization, and that children living with HIV with CoV infections develop more severe outcomes.

**IMPORTANCE** SARS-CoV-2 may cause severe hospitalization, but little is known about the role of secondary bacterial infection in these severe cases, beyond the observation of high levels of reported inflammatory markers, associated with bacterial infection, such as procalcitonin. We did a secondary analysis of a double-blind randomized trial of PCV to examine its impact on human CoV infections before the pandemic. We found that both children living with and without HIV randomized to receive PCV had evidence of less hospitalization due to seasonal CoV, suggesting that pneumococcal coinfection may play a role in severe hospitalized CoV infections.

**KEYWORDS** coronaviruses, HIV, pneumonia, respiratory infections, PCV9, pneumococcal vaccine, pneumococcal conjugate vaccine, SARS-CoV-2, *Streptococcus pneumoniae*, human coronavirus, human immunodeficiency virus

Lower respiratory tract infections (LRTI) in young children are a major cause of hospital admissions (1). Globally, a large proportion of pneumonia hospitalizations are attributed to respiratory viral infections, including 61.4% in the multisite, international case-control PERCH (Pneumonia Etiology Research for Child Health) study undertaken

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in seven low- and middle-income countries among children aged 1 to 59 months (2). Coronaviruses (CoVs) are enveloped, nonsegmented, positive-sense, single-stranded RNA viruses and are associated with various natural hosts. Four CoVs are currently endemic in the human population, CoV-OC43, CoV-229E, CoV-NL63, and CoV-HKU1. CoV-OC43 and CoV-229E were initially identified as causes of upper respiratory tract infections in the 1960s using classical culture methods (3, 4). More recently, advances in molecular diagnostics have resulted in the identification of CoV-NL63 (5) and CoV-HKU1 (6). Infections by these endemic CoVs may involve the upper respiratory tract and the gastrointestinal tract; infections range from being completely asymptomatic to causing mild, self-limiting disease and, less commonly, severe illness such as bronchitis and pneumonia (7). Interestingly, hospitalized children with CoV-associated LRTI more frequently had coinfections with respiratory syncytial virus (RSV) and a higher abundance of *Haemophilus influenzae* than children hospitalized for non-CoV-associated LRTI or asymptomatic healthy CoV carriers (8), suggesting that viral and bacterial coinfections might trigger severe CoV infections.

Three other CoVs have, however, caused outbreaks of deadly pneumonia in humans since the beginning of the 21st century. The severe acute respiratory syndrome coronavirus (SARS-CoV), emerging in 2002, was responsible for an epidemic that spread to five continents with an overall fatality rate of approximately 10% with no fatalities reported in children (5), the Middle East respiratory syndrome coronavirus (MERS-CoV) has caused recurrent outbreaks in humans since 2012 mostly in the Arabian Peninsula with a fatality rate of 20% to 40% in adults and about 6% in children (9, 10), and the recent SARS-CoV-2 that appeared first in China at the end of 2019 has since caused a global pandemic (11). Although progress is rapidly being made, no specific antiviral vaccines are available to prevent any human CoV infections. Understanding the epidemiology and morbidity associated with common human-adapted CoVs may assist in informing treatment and mitigation strategies for severe illness from novel CoVs of zoonotic origin, although similar viruses may undergo different selective pressures in order to maintain fitness and host adaptation (12), making viral transmission patterns across species and their interactions with other potential coinfecting or superinfecting pathogens hard to predict (13).

Due to impaired humoral and cell-mediated immunity, children living with HIV (more so if not on antiretroviral treatment) have an increased risk for severe illness and mortality from virus-associated LRTI, including those due to RSV and influenza virus (14, 15). There are, however, limited data on the role of other respiratory viruses, including CoVs in children hospitalized with pneumonia, and only a few studies with limited numbers specifically included participants living with HIV (16, 17). A study in the United States involving immunocompromised children, the majority of whom had hematologic malignancy or solid tumor, found that these children were at higher risk of CoV-associated LRTI that necessitated oxygen use than were nonimmunocompromised children. In that study, younger age, underlying pulmonary disorder, and the concomitant presence of RSV were also associated with severe CoV-associated LRTI (18).

The aim of this study was to determine the burden and clinical features of CoV infections in children living with and without HIV hospitalized for pneumonia. Furthermore, as we have previously done for other respiratory viruses (19–21), we probed, using a randomized, placebo-controlled trial of an investigational 9-valent pneumococcal conjugate vaccine (PCV9), whether PCV9 could prevent CoV-associated hospitalization and thereby infer that pneumococcal infection may contribute to hospitalization for CoV-associated pneumonia.

## RESULTS

During the follow-up period included in this analysis, from all the participants in the initial trial, 347 (162 PCV9 group and 185 placebo recipients) children living with HIV and 448 (202 PCV9 group and 246 placebo recipients) without HIV had at least one

**TABLE 1** Prevalence of human coronavirus-associated pneumonia in children 1 to 23 months old living with and without HIV

|   | No. (%)              |                              |                                 | P value <sup>b</sup> |
|---|----------------------|------------------------------|---------------------------------|----------------------|
|   | Overall<br>(n = 750) | Living with<br>HIV (n = 347) | Living without<br>HIV (n = 448) |                      |
| CoV-OC43                                | 68 (8.6)             | 56 (16.1)                    | 12 (3.7)                        | <0.001               |
| CoV-NL63                                | 22 (2.8)             | 8 (2.3)                      | 14 (3.1)                        | 0.48                 |
| CoV-HKU1                                | 22 (2.8)             | 13 (3.8)                     | 9 (2.0)                         | 0.14                 |
| CoV-229E                                | 1 (0.1)              | 0                            | 1 (0.2)                         | 0.99                 |
| At least one CoV infection <sup>a</sup> | 103 (13.0)           | 69 (19.9)                    | 34 (7.6)                        | <0.001               |

<sup>a</sup>Some children had coinfection by two CoVs, and some had multiple episodes of CoV-associated pneumonia.

<sup>b</sup>P values adjusted for study group, i.e., placebo or pneumococcal conjugate vaccine.

episode of pneumonia tested for CoV infection. Similar proportions of samples were tested from PCV9 recipients and placebo recipients (22). Of the tested children, 69 (19.9%) living with HIV and 34 (7.6%;  $P < 0.001$ ) without HIV tested CoV PCR positive (Table 1).

Five children living with HIV had the same virus detected for long periods, including three children with CoV-OC43 detected 32, 38, and 70 days apart; one child with CoV-HKU1 identified twice 74 days apart; and one child in whom CoV-OC43 was identified twice 219 days apart. One child living with HIV had three CoV-associated admissions; the child initially tested positive for CoV-OC43, then 5 months later CoV-NL63 was detected, and after 3 months CoV-OC43 was detected again. Six children living with HIV had two distinct CoVs identified at two different admissions.

When assessing the CoV-associated pneumonia episodes among children randomized to placebo, children living with HIV compared to those without HIV were more likely to have oxygen saturation of <90% and stay in hospital for longer than 2 days. The percentage of CoV-associated pneumonia episodes with CoVs detected as single viral infection was similar among placebo recipients living with HIV compared to those without HIV (Table 2).

Bacterial cultures were performed in 94.8% of CoV-associated pneumonia admissions. *Streptococcus pneumoniae* was isolated from blood specimens of two children living with HIV collected at the same admission at which CoV-NL63 was detected in nasopharyngeal aspirate (NPA), one PCV9 recipient (serotype 23F) and one placebo recipient (serotype 6A).

Considering children with CoV-associated pneumonia, those living with HIV had a higher case fatality risk (30.4%,  $n = 21$ ) than children without HIV (2.9%,  $n = 1$ ;  $P = 0.001$ ). This was similar to the case fatality risks for children hospitalized for pneumonia in whom CoV infections were not detected (27.0% living with HIV versus 1.7% without HIV). The median age of the 21 children living with HIV who died was 3 months (range: 1 to 15 months) (Table 3). CoV-OC43 was the only virus detected in eight of the fatal cases. A coinfection of CoV-OC43 and CoV-NL63 was detected in a 2-month-old fatal case. Among children living with HIV, the case fatality risk in those with episodes where CoVs were the only viruses detected (32.1%) was similar to that in episodes with coinfections by CoVs and other viruses (22.6%;  $P = 0.35$ ). *Escherichia coli* was isolated from blood of three children (all living with HIV), and *Pneumocystis jirovecii* infection was identified in 10 (62.5%) children with HIV. Seven (36.8%) of the children living with HIV who died had levels of C-reactive protein of  $\geq 40$  mg/liter, and 11 (64.7%) had procalcitonin levels of  $\geq 0.5$  ng/ml (Table 3).

Overall, PCV9 recipients had a 33.9% (95% CI: 2.0% to 55.4%) lower risk for CoV-associated pneumonia hospitalizations than placebo recipients. In children living without HIV, PCV9 vaccination was associated with a 64.0% (95% CI: 22.9% to 83.2%) reduction in hospitalization with CoV-associated pneumonia, including reductions of 90.9% (95% CI: 29.5% to 98.8%) and 87.5% (95% CI: 0.1% to 98.4%) for CoV-OC43- and CoV-

**TABLE 2** Demographic, clinical, and laboratory characteristics of children living with and without HIV hospitalized with coronavirus-associated pneumonia<sup>e</sup>

|   | Living with HIV |                 | Living without HIV |                | P value <sup>a</sup> |
|---|-----------------|-----------------|--------------------|----------------|----------------------|
|   | Placebo         | PCV             | Placebo            | PCV            |                      |
| Specimens tested                                | 252             | 207             | 256                | 212            | -                    |
| CoV-positive samples                            | <b>44</b>       | <b>37</b>       | <b>25</b>          | <b>9</b>       | -                    |
| CoV detected as single viral infections         | 17 (38.6)       | 10 (27.0)       | 8 (32.0)           | 4 (44.4)       | 0.91 <sup>b</sup>    |
| Mean age in mo (SD)                             | 8.9 (6.3)       | 9.5 (6.4)       | 11.7 (6.1)         | 10.4 (6.4)     | 0.10                 |
| Axillary temp $\geq 38^{\circ}\text{C}$         | 8 (42.1) [19]   | 4 (18.2) [22]   | 3 (23.1) [13]      | 0 [7]          | 0.47                 |
| Mean respiratory rate, breaths per minute (SD)  | 57.5 (14.9)     | 56.3 (13.2)     | 51.1 (12.6)        | 58.7 (14.1)    | 0.076                |
| Cyanosis  | 9 (21.4) [42]   | 13 (35.1)       | 2 (8.0)            | 1 (11.1)       | 0.34                 |
| Oxygen saturation on room air $< 90\%$          | 24 (54.6)       | 16 (43.2)       | 5 (20.0)           | 3 (33.3)       | <b>0.013</b>         |
| Bronchial breathing                             | 10 (22.7)       | 7 (18.9)        | 2 (8.0)            | 1 (11.1)       | 0.08                 |
| Hospital stay $> 2$ days                        | 32 (76.2) [42]  | 27 (73.0)       | 18 (75.0) [24]     | 5 (55.6)       | 0.54                 |
| History of fever                                | 18 (41.9) [43]  | 15 (40.5)       | 11 (44.4)          | 3 (33.3)       | 0.66                 |
| History of vomiting                             | 0 [42]          | 2 (5.4)         | 2 (8.0)            | 0              | 0.14 <sup>d</sup>    |
| History of seizures                             | 17 (46.0) [37]  | 13 (41.9) [31]  | 9 (36.0)           | 2 (25.0) [8]   | 0.25                 |
| Alveolar consolidation on chest X-ray           | 10 (22.7)       | 7 (18.9)        | 2 (8.0)            | 1 (11.1)       | 0.08                 |
| C-reactive protein $\geq 40$ mg/liter           | 15 (44.1) [34]  | 12 (40.0) [30]  | 7 (35.0) [20]      | 3 (42.9) [7]   | 0.19                 |
| Procalcitonin $\geq 2$ ng/ml                    | 6 (30.0) [20]   | 10 (40.0) [25]  | 5 (27.8) [18]      | 2 (33.3) [6]   | 0.49                 |
| Mean white cell count cells, $10^9$ /liter (SD) | 12.8 (6.0) [13] | 11.6 (6.7) [11] | 23.1 (15.8) [6]    | 12.6 (7.2) [2] | 0.14                 |
| Bacterial infection <sup>c</sup>                | 2 (5.1) [39]    | 6 (16.7) [36]   | 1 (4)              | 0              | 0.98                 |

<sup>a</sup>P values compare the placebo group between children living with and without HIV, adjusted for age at hospitalization, detection of previously tested viruses, and year of collection. Values in bold indicate statistically significant comparisons.

<sup>b</sup>P values compare the placebo group between children living with and without HIV, adjusted for age at hospitalization, and year of collection.

<sup>c</sup>Blood specimens were available for culture from 109 coronavirus-associated pneumonia episodes, 75 HIV infected and 34 HIV uninfected. Bacteria isolated from children living with HIV included *Streptococcus pneumoniae* ( $n = 2$ ), *Escherichia coli* ( $n = 3$ ), *Salmonella* sp. ( $n = 1$ ), *Streptococcus viridans* ( $n = 1$ ), and other *Streptococcus* ( $n = 1$ ). Bacteria isolated from children without HIV included *Streptococcus viridans* ( $n = 1$ ) and *Micrococcus* ( $n = 1$ ).

<sup>d</sup>P value calculated by Fisher's exact test.

<sup>e</sup>Results are presented as number of participants with a particular presentation and percentage in parentheses, unless otherwise indicated. Numbers in brackets are number of participants with available information if different from total. PCV, pneumococcal conjugate vaccine. SD, standard deviation.

HKU1-associated hospitalizations, respectively. No vaccine efficacy was observed for pneumonia episodes without viruses identified (Table 4). No significant protection of PCV9 against hospitalization for CoV-associated pneumonia was observed in children living with HIV (Table 4).

## DISCUSSION

The results of this study suggest a possible interaction between endemic human CoV infection and *Streptococcus pneumoniae* in children without HIV (23). In the context of a double-blind placebo-randomized trial, we demonstrated that at least 34% of children hospitalized for CoV-associated pneumonia were also probably coinfecting with *Streptococcus pneumoniae* which might have precipitated their hospitalization. This is supported by *in vitro* experiments that have shown that CoV-NL63 infection resulted in an increased adherence of *Streptococcus pneumoniae* to virus-infected epithelial cells (24). The imputed rate of coinfection with pneumococci in children with CoV-associated pneumonia from our study provides a conservative estimate of this possible interaction as only 9 pneumococcal serotypes were included in the vaccine and vaccine efficacy, even against vaccine-serotype pneumococcal pneumonia, is not 100%. The high calculated vaccine efficacy estimates for CoV-OC43- and CoV-HKU1-associated disease needs to be contextualized within the wide uncertainty bounds of this estimate. Furthermore, we report on the prevalence and clinical features of endemic CoV-associated pneumonia in children living with and without HIV. CoVs were detected among 19.9% of the hospitalized children living with HIV and in 7.6% of children without HIV. CoV-associated mortality was 30.4% in children living with HIV with 38.1% of these fatal cases having CoV-OC43 as the only virus identified; mortality rates did not differ between children with CoV detected as single infections and those with other viral coinfections.

Although our study was not designed to establish whether CoV infections caused more severe disease in children living with HIV, among children with CoV-associated



**TABLE 3** Characteristics of the 22 children who died during hospitalization with coronavirus-associated pneumonia

| Age at hospitalization (mo) | Gender | Living with HIV | Diagnosis | Study group | Virus(es) detected <sup>a</sup>    | Chest X-ray result     | CRP (mg/liter) | PCT (ng/ml) | Bacteria isolated from blood | <i>P. jirovecii</i> infection |
|-----------------------------|--------|-----------------|-----------|-------------|------------------------------------|------------------------|----------------|-------------|------------------------------|-------------------------------|
| 1                           | Male   | Yes             | Pneumonia | PCV         | CoV-OC43 + influenza               | Normal                 | 270            | 14.9        | No                           | Not tested                    |
| 2                           | Female | Yes             | Pneumonia | PCV         | CoV-OC43 + hRV                     | Normal                 | 7              | 0.1         | No                           | Yes                           |
| 2                           | Female | Yes             | Pneumonia | PCV         | CoV-OC43                           | Alveolar consolidation | 14             | 0.2         | No                           | Yes                           |
| 2                           | Male   | Yes             | Pneumonia | PCV         | CoV-OC43                           | Alveolar consolidation | 1              | 0.2         | No                           | Yes                           |
| 3                           | Male   | Yes             | Pneumonia | PCV         | CoV-OC43 + hBoV                    | Normal                 | 1              | 4.4         | No                           | No                            |
| 4                           | Female | Yes             | Pneumonia | PCV         | CoV-OC43 + hBoV                    | Normal                 | 127            | 1.2         | No                           | Yes                           |
| 4                           | Male   | Yes             | Pneumonia | PCV         | CoV-OC43                           | Alveolar consolidation | 3              | Unknown     | No                           | Yes                           |
| 9                           | Male   | Yes             | Pneumonia | PCV         | CoV-OC43                           | Unknown                | 2              | 8.8         | No                           | No                            |
| 10                          | Female | Yes             | Pneumonia | PCV         | CoV-OC43 + WUPyV + hRV + influenza | Unknown                | 210            | 174.9       | <i>Escherichia coli</i>      | Not tested                    |
| 14                          | Male   | Yes             | Pneumonia | PCV         | CoV-OC43 + KIPyV                   | Alveolar consolidation | 126            | 2.4         | <i>Escherichia coli</i>      | Yes                           |
| 2                           | Male   | Yes             | Pneumonia | Placebo     | CoV-OC43 + CoV-NL63                | Normal                 | 92             | 6.2         | No                           | Not tested                    |
| 2                           | Male   | Yes             | Pneumonia | Placebo     | CoV-OC43 + hRV                     | Normal                 | 1              | 0.2         | No                           | Yes                           |
| 2                           | Female | Yes             | Pneumonia | Placebo     | CoV-OC43 + hRV                     | Unknown                | 6              | 0.1         | No                           | Not tested                    |
| 2                           | Male   | Yes             | Pneumonia | Placebo     | CoV-OC43 + KIPyV                   | Alveolar consolidation | 21             | 0.6         | No                           | Yes                           |
| 3                           | Male   | Yes             | Pneumonia | Placebo     | CoV-OC43                           | Normal                 | 2              | 0.1         | Not performed                | No                            |
| 3                           | Female | Yes             | Pneumonia | Placebo     | CoV-OC43 + hRV                     | Alveolar consolidation | 39             | Unknown     | No                           | No                            |
| 5                           | Female | Yes             | Pneumonia | Placebo     | CoV-OC43                           | Unknown                | Unknown        | Unknown     | Not performed                | No                            |
| 7                           | Female | Yes             | Pneumonia | Placebo     | CoV-OC43                           | Normal                 | Unknown        | Unknown     | No                           | Not tested                    |
| 8                           | Female | Yes             | Pneumonia | Placebo     | CoV-OC43 + hBoV                    | Normal                 | 218            | 302.0       | <i>Escherichia coli</i>      | Yes                           |
| 11                          | Female | Yes             | Pneumonia | Placebo     | CoV-HKU1 + hRV + KIPyV + influenza | Alveolar consolidation | 37             | 0.66        | No                           | Yes                           |
| 15                          | Female | Yes             | Pneumonia | Placebo     | CoV-OC43                           | Alveolar consolidation | 464            | 254.7       | No                           | No                            |
| 3                           | Female | No              | Pneumonia | Placebo     | CoV-229E + KIPyV                   | Unknown                | 1              | Unknown     | No                           | Yes                           |

<sup>a</sup>Including viruses detected by immunofluorescence assay (influenza virus, respiratory syncytial virus [RSV], parainfluenza viruses [PIV], and adenovirus), nested PCR (human metapneumovirus), and PCR (human bocavirus [hBoV]; human rhinovirus [hRV]; human coronavirus [CoV]-OC43, -NL63, -HKU1, and -229E; and polyomaviruses WU [WUPyV] and KI [KIPyV]). PCV, pneumococcal conjugate vaccine; CRP, C-reactive protein; PCT, procalcitonin.

**TABLE 4** Differences in incidence of coronavirus-associated pneumonia episodes between immunized children who received 9-valent pneumococcal conjugate vaccine and placebo recipients, intent-to-treat analysis

|  | All children                     |                         |                              | Children living without HIV |                     |                         | Children living with HIV     |         |                    |                        |                              |         |
|--|----------------------------------|-------------------------|------------------------------|-----------------------------|---------------------|-------------------------|------------------------------|---------|--------------------|------------------------|------------------------------|---------|
|  | PCV <sup>c</sup><br>(n = 19,922) | Placebo<br>(n = 19,914) | Vaccine efficacy<br>(95% CI) | P value                     | PCV<br>(n = 18,633) | Placebo<br>(n = 18,626) | Vaccine efficacy<br>(95% CI) | P value | PCV<br>(n = 1,289) | Placebo<br>(n = 1,288) | Vaccine efficacy<br>(95% CI) | P value |
| CoV-OC43                                       | 27 (0.1)                         | 41 (0.2)                | 34.2 (-7.0, 59.5)            | 0.09                        | 1 (0.01)            | 11 (0.06)               | 90.9 (29.5, 98.8)            | 0.004   | 26 (2.0)           | 30 (2.3)               | 13.4 (-45.6, 48.5)           | 0.60    |
| CoV-NL63                                       | 12 (0.06)                        | 10 (0.05)               | -20.0 (-177.6, 48.1)         | 0.67                        | 8 (0.04)            | 6 (0.03)                | -33.3 (-284.1, 53.7)         | 0.59    | 4 (0.3)            | 4 (0.3)                | 0.08 (-2,986.7, 75.0)        | 0.99    |
| CoV-HKU1                                       | 6 (0.03)                         | 16 (0.08)               | 62.5 (4.2, 85.3)             | 0.033                       | 1 (0.01)            | 8 (0.04)                | 87.5 (0.1, 98.4)             | 0.020   | 5 (0.4)            | 8 (0.6)                | 37.5 (-90.4, 79.5)           | 0.40    |
| Any CoV <sup>a</sup>                           | 41 (0.2)                         | 62 (0.3)                | 33.9 (2.0, 55.4)             | 0.038                       | 9 (0.05)            | 25 (0.1)                | 64.0 (22.9, 83.2)            | 0.006   | 32 (2.5)           | 37 (2.9)               | 13.6 (-37.8, 45.8)           | 0.54    |
| CoV detected as single<br>viral infections     | 14 (0.07)                        | 23 (0.1)                | 39.2 (-18.2, 68.7)           | 0.14                        | 4 (0.02)            | 8 (0.04)                | 50.0 (-66.0, 84.9)           | 0.25    | 10 (0.8)           | 15 (1.2)               | 33.4 (-47.7, 70.0)           | 0.31    |
| All-cause pneumonia <sup>a</sup>               | 518 (2.6)                        | 612 (3.1)               | 15.4 (5.0, 24.6)             | 0.004                       | 318 (1.7)           | 369 (2.0)               | 13.9 (0.05, 25.7)            | 0.049   | 200 (15.5)         | 243 (18.9)             | 17.8 (2.4, 30.6)             | 0.024   |
| Any virus-associated<br>pneumonia <sup>b</sup> | 304 (1.5)                        | 389 (2.0)               | 21.9 (9.4, 32.7)             | 0.001                       | 190 (1.0)           | 252 (1.4)               | 24.6 (9.1, 37.5)             | 0.003   | 114 (8.8)          | 114 (8.8)              | 16.9 (-5.3, 34.3)            | 0.13    |
| Pneumonia without<br>viruses identified        | 239 (1.2)                        | 255 (1.3)               | 6.3 (-11.6, 21.4)            | 0.47                        | 125 (0.7)           | 120 (0.6)               | -4.1 (-33.7, 18.9)           | 0.75    | 114 (8.8)          | 135 (10.5)             | 15.6 (-6.9, 33.4)            | 0.16    |

<sup>a</sup>First episode: children with pneumonia episodes associated with different coronaviruses are counted in that virus for each first episode but only once in any coronavirus. Children with pneumonia episodes associated both with and without a virus are counted in that category for each first episode but only once in the total number of all-cause pneumonia.

<sup>b</sup>Includes all viruses investigated: influenza virus, respiratory syncytial virus, parainfluenza viruses, adenovirus, human metapneumovirus, human bocavirus, human rhinovirus, coronaviruses, and polyomaviruses WU and KI.

<sup>c</sup>PCV, pneumococcal conjugate vaccine.

hospitalization, those living with HIV were more likely to have longer duration of hospitalization and a higher case fatality rate than children without HIV, similarly to what was previously described for other viral infections (14, 19, 25). The increased morbidity and mortality in children living with HIV could have been, however, due to increased susceptibility to pneumococcal and nonpneumococcal coinfections, such as *P. jirovecii*, *Escherichia coli*, *Haemophilus influenzae*, or other viral infections (8, 18). Other respiratory viruses were, however, not found at higher rates in children living with HIV infected with CoV compared to those without HIV. The high rate of *P. jirovecii* detection in fatal cases in children living with HIV also suggests that CoVs may lead to secondary *P. jirovecii* activation. Nonetheless, the higher case fatality rate and the fact that all eight fatal cases with CoV as the only respiratory virus detected were children living with HIV suggest a possible association of CoV causing severe disease in these children.

In the current study, of the children living with HIV who died, 36.8% and 64.7% had high levels of C-reactive protein and procalcitonin, respectively. Elevated levels of C-reactive protein and procalcitonin are normally detected in patients with bacterial infections compared to those with viral infections alone (26). However, high levels of these two markers may also be suggestive of unrecognized bacterial coinfections in patients with established viral etiology for pneumonia (27). Recent reports have described clear associations for raised C-reactive protein and procalcitonin levels with severity in patients with SARS-CoV-2 infection (28), including in fatal SARS-CoV-2 confirmed cases (29), further underscoring the complexity of host-novel pathogen interaction. Our results together with other data from seasonal CoVs (8) and SARS-CoV-2 suggest a role in bacterial coinfection in those CoV-infected patients who develop severe forms of disease.

Recurrent CoV-associated pneumonia episodes were detected in children living with HIV. This could be the result of extended viral shedding since these children have compromised cell-mediated immunity, which is necessary for termination of viral shedding, or due to viral reactivation or reinfection. Nonetheless, in these children initial immune response to infection was not enough to stop infection. Under the current SARS-CoV-2 pandemic, studies of extended viral shedding among children living with HIV are warranted.

To date, there are only a few reports on secondary or superinfections in SARS-CoV-2 patients. A meta-analysis of 28 studies found that the overall bacterial infections in SARS-CoV-2 patients were 7.1%, suggesting that bacterial coinfections are relatively uncommon (30); the bacteriological testing method used was, however, not specified in the majority of the studies (14 studies), and four studies used blood culture, a known poorly sensitive method to detect bacterial infections. These results do not discard a role for pneumococcal superinfections, particularly in PCV-underserved settings, as a report from Spain of five SARS-CoV-2 patients showed coinfection with *Streptococcus pneumoniae* (31). The findings of these recent studies relative to our study underscore the complexity of extrapolating from established human-adapted viruses with relatively low case fatality rates to a novel zoonotic virus with a relatively high case fatality rate that has been thus far predominately described as a primary viral pneumonitis. Another restriction of using endemic CoV as a surrogate for SARS-CoV-2 is that the tropism of the endemic viruses is mainly the upper respiratory tract while SARS-CoV-2 has propensity for both the upper and lower respiratory tract.

CoVs were normally detected in conjunction with other respiratory viruses in both children living with and those living without HIV, which is similar to previous reports (32). Considering all the respiratory viruses tested in the current set of samples, there were no differences in the frequency of multiple detections in PCV9 recipients (26%) compared to placebo recipients (27%) in both children living with (25% versus 21%) and without (27% versus 30%) HIV (22). In the absence of a control group of non-LRTI children, the relationship between CoV infection and disease cannot, however, be definitively established, and this constitutes a limitation of our study. Actually, a case-

control study on the etiology of childhood pneumonia in South Africa and the PERCH study were unable to attribute the detection of endemic CoV as causes of pneumonia requiring hospital admission, since the prevalence of CoV infection was similar in cases and controls (2, 33). The PERCH study was, however, not designed to quantify the importance of coinfections, and caution is needed to directly compare those findings with our observations where interactions with *Streptococcus pneumoniae* might have led CoV to be associated with pneumonia. Our results suggest that CoV infection likely predisposes to superimposed bacterial infections, as previously shown for other respiratory viruses, so although not directly attributable as the cause of pneumonia, as shown by the previous studies using a single-pathogen model approach, it does not exclude CoVs from being in the causal pathway of the disease. An alternate explanation could be that if, for instance, other respiratory viruses interfere with CoV infection, CoV would appear more frequently in the PCV9 group, even in the absence of a biological interaction with *Streptococcus pneumoniae*. Our vaccine efficacy point estimates for CoV single infection-associated pneumonia and overall CoV-associated pneumonia were very similar, suggesting an interaction between *Streptococcus pneumoniae* and CoV in the absence of other viral infections.

In the hypothetical scenario of a perfect vaccine against CoV, it would be interesting to explore this key question on whether there is a biological interaction between CoV and *Streptococcus pneumoniae* by investigating if the incidence or severity of pneumococcal pneumonia would be affected by the viral vaccine.

A limitation of our study is that the children living with HIV in this cohort were not treated with antiretroviral treatment due to lack of availability for children in the public sector in South Africa 20 years ago, which may have contributed to their clinical course and may differ from that of HIV-infected children currently treated with antiretrovirals. The fact that the study was single-center and that children enrolled into the trial might access health care differently than the general population limits the generalization of the results to other settings.

CoVs are classified into four groups, alpha, beta, gamma, and delta; only alpha and beta CoVs are known to infect humans. The beta group is further composed of A, B, C, and D subgroups (34). While CoV-229E and CoV-NL63 are alpha viruses, CoV-OC43, the most common CoV identified in our study, and CoV-HKU1, together with MERS-CoV, SARS-CoV, and SARS-CoV-2, belong to the beta group (35). Although seroprevalence studies suggest that exposure to the four common CoVs is widespread during childhood and that approximately 90% of adults are seropositive for at least one CoV (36), this humoral immunity may not be cross protective against the novel CoV species. *In vitro* studies have, however, shown that CD4<sup>+</sup> T cells from SARS-CoV-2-naïve donors responded not only to common CoVs but also to SARS-CoV-2, suggesting possible cross-reactivity (37, 38). The role of preexisting SARS-CoV-2 cross-reactive T cells in clinical outcomes remains to be determined.

It has been suggested that infection by SARS-CoV-2 in children causes milder clinical symptoms than in adults (39). In this analysis we show that pneumococcal conjugate vaccine protected children living without HIV against CoV-associated pneumonia hospitalization, and this preventive strategy should be explored in the current pandemic.

## MATERIALS AND METHODS

**Population.** We undertook a *post hoc* analysis of children who participated in an efficacy trial of PCV9 in South Africa as described previously (20, 23). Briefly, 39,836 children were recruited from March 1998 to October 2000 and randomized (1:1) to receive 3 doses (6, 10, and 14 weeks of age) of either PCV9 or placebo, including 2,577 children living with HIV and 37,259 without HIV (23). Hospital-based surveillance for all-cause hospitalization among study participants was undertaken until October 2006. All hospitalized children were evaluated by study doctors and underwent HIV testing according to study protocol (23). Nasopharyngeal aspirates (NPAs) were obtained from children hospitalized with LRTI for identification of select respiratory viruses (20) and archived from February 2000 onward. In the present study, only NPAs collected from 1 February 2000 to 31 January 2002 from children <2 years old admitted with pneumonia diagnosis (defined as presence of alveolar consolidation on chest X-ray, or clinical diagnosis of LRTI without wheeze on chest auscultation, but with rales and/or bronchial breathing) were

analyzed. If a child had recurrent pneumonia hospitalizations, only NPAs collected at least 28 days apart or with different viruses detected were included in the analysis.

**Viral testing.** Samples were investigated using immunofluorescence assay for RSV, influenza virus, parainfluenza viruses (PIV) I to III and adenovirus and by nested PCR for human metapneumovirus as described previously (20, 21); real-time reverse transcriptase PCR was later performed to detect four human CoVs, OC43, NL63, HKU1, and 229E, as well as human bocavirus, human rhinovirus (hRV), and WU and KI polyomaviruses using the primers and probes as previously detailed (22). In this analysis, we describe the epidemiology of CoV-associated pneumonia in the study cohort, including exploring differences in hospitalization rates between those who were randomized to receive PCV9 and those who received placebo. We have previously used this randomized placebo-controlled trial as a probe to establish that by protecting against pneumococcal disease, there was also a decrease in hospitalizations for virus-associated pneumonia, specifically influenza virus-, RSV-, human metapneumovirus-, and polyomavirus-associated pneumonia (19–21). The rationale of this approach is that the difference in incidence of any disease between PCV9 recipients and placebo recipients that could potentially be associated with vaccine-serotype pneumococcal infection would indicate a role of vaccine serotypes (40). As an example, we have previously reported that PCV9 recipients had a 45% lower incidence of hospitalization for pneumonia in which influenza virus was identified, and as such we concluded that at least a similar proportion of the influenza-associated pneumonias among the placebo recipients was precipitated by coinfection with PCV9 serotypes (20). This is particularly important considering the lack of a sensitive diagnostic tool to diagnose bacterial pneumonia, including the sensitivity of blood culture being only <5% for diagnosing pneumococcal pneumonia in children (41).

**Statistical analysis.** Regression analyses were performed to compare the prevalence of CoVs and the clinical features between children living with and without HIV. Multiple regressions were controlled for age at hospitalization, detection of a virus previously tested, and year of sample collection. Using the concept of vaccine-probe studies (20), we explored whether there was any association between receipt of PCV9 and the risk of hospitalization for pneumonia associated with CoV infection by estimating vaccine efficacy (VE) based on the formula

$$VE (\%) = \frac{\text{incidence rate in the unvaccinated} - \text{incidence rate in the vaccinated}}{\text{incidence rate in the unvaccinated}} \times 100$$

All randomized children were included in the intent-to-treat analysis from the day they received their first dose of study vaccine. The first episode was included in the VE calculation for an individual participant, and *P* values of  $\leq 0.05$  were considered significant. Analyses were performed using STATA version 13.1 (College Station, TX, USA).

**Ethical considerations.** The main efficacy trial and subsequent *post hoc* analyses were approved by the Human Research Ethics Committee (Medical) of the University of the Witwatersrand. Signed written informed consent was obtained from the parents/legal guardians as part of the trial. The Ethics Committee did not require additional consent for this analysis. The main study was not registered under any clinical trial registry as it was undertaken prior to registration being made mandatory.

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Declaration of interests: nothing to declare.

## REFERENCES

- Nair H, Simoes EA, Rudan I, Gessner BD, Azziz-Baumgartner E, Zhang JS, Feikin DR, Mackenzie GA, Moïsi JC, Roca A, Baggett HC, Zaman SM, Singleton RJ, Lucero MG, Chandran A, Gentile A, Cohen C, Krishnan A, Bhutta ZA, Arguedas A, Clara AW, Andrade AL, Ope M, Ruvinsky RO, Hortal M, McCracken JP, Madhi SA, Bruce N, Qazi SA, Morris SS, El Arifeen S, Weber MW, Scott JAG, Brooks WA, Breiman RF, Campbell H, Severe Acute Lower Respiratory Infections Working Group. 2013. Global and regional burden of hospital admissions for severe acute lower respiratory infections in young children in 2010: a systematic analysis. *Lancet* 381:1380–1390. [https://doi.org/10.1016/S0140-6736\(12\)61901-1](https://doi.org/10.1016/S0140-6736(12)61901-1).
- Pneumonia Etiology Research for Child Health (PERCH) Study Group. 2019. Causes of severe pneumonia requiring hospital admission in children without HIV infection from Africa and Asia: the PERCH multi-country case-control study. *Lancet* 394:757–779. [https://doi.org/10.1016/S0140-6736\(19\)30721-4](https://doi.org/10.1016/S0140-6736(19)30721-4).
- Hamre D, Procknow JJ. 1966. A new virus isolated from the human respiratory tract. *Proc Soc Exp Biol Med* 121:190–193. <https://doi.org/10.3181/00379727-121-30734>.
- McIntosh K, Dees JH, Becker WB, Kapikian AZ, Chanock RM. 1967. Recovery in tracheal organ cultures of novel viruses from patients with respiratory disease. *Proc Natl Acad Sci U S A* 57:933–940. <https://doi.org/10.1073/pnas.57.4.933>.

5. van der Hoek L, Pyrc K, Jebbink MF, Vermeulen-Oost W, Berkhout RJM, Wolthers KC, Wertheim-van Dillen PME, Kaandorp J, Spaargaren J, Berkhout B. 2004. Identification of a new human coronavirus. *Nat Med* 10:368–373. <https://doi.org/10.1038/nm1024>.
6. Woo PCY, Lau SKP, Tsoi H-W, Huang Y, Poon RWS, Chu C-M, Lee RA, Luk W-K, Wong GKM, Wong BHL, Cheng VCC, Tang BSF, Wu AKL, Yung RWH, Chen H, Guan Y, Chan K-H, Yuen K-Y. 2005. Clinical and molecular epidemiological features of coronavirus HKU1-associated community-acquired pneumonia. *J Infect Dis* 192:1898–1907. <https://doi.org/10.1086/497151>.
7. Kenmoe S, Tchendjou P, Vernet M-A, Moyo-Tetang S, Mossus T, Njankou-Ripa M, Kenne A, Penlap Beng V, Vabret A, Njouom R. 2016. Viral etiology of severe acute respiratory infections in hospitalized children in Cameroon, 2011–2013. *Influenza Other Respir Viruses* 10:386–393. <https://doi.org/10.1111/iv.12391>.
8. de Koff EM, van Houten MA, Sanders EAM, Bogaert D. 2021. Severity of respiratory infections with seasonal coronavirus is associated with viral and bacterial coinfections. *Pediatr Infect Dis J* 40:e36–e39. <https://doi.org/10.1097/INF.0000000000002940>.
9. Zaki AM, van Boheemen S, Bestebroer TM, Osterhaus AD, Fouchier RA. 2012. Isolation of a novel coronavirus from a man with pneumonia in Saudi Arabia. *N Engl J Med* 367:1814–1820. <https://doi.org/10.1056/NEJMoa1211721>.
10. Zimmermann P, Curtis N. 2020. Coronavirus infections in children including COVID-19: an overview of the epidemiology, clinical features, diagnosis, treatment and prevention options in children. *Pediatr Infect Dis J* 39:355–368. <https://doi.org/10.1097/INF.0000000000002660>.
11. Chen N, Zhou M, Dong X, Qu J, Gong F, Han Y, Qiu Y, Wang J, Liu Y, Wei Y, Xia J, Yu T, Zhang X, Zhang L. 2020. Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *Lancet* 395:507–513. [https://doi.org/10.1016/S0140-6736\(20\)30211-7](https://doi.org/10.1016/S0140-6736(20)30211-7).
12. Ilyushina NA, Bovin NV, Webster RG. 2012. Decreased neuraminidase activity is important for the adaptation of H5N1 influenza virus to human airway epithelium. *J Virol* 86:4724–4733. <https://doi.org/10.1128/JVI.06774-11>.
13. Herfst S, Mok CKP, van den Brand JMA, van der Vliet S, Rosu ME, Spronken MI, Yang Z, de Meulder D, Lexmond P, Bestebroer TM, Peiris JSM, Fouchier RAM, Richard M. 2018. Human clade 2.3.4.4 A/H5N6 influenza virus lacks mammalian adaptation markers and does not transmit via the airborne route between ferrets. *mSphere* 3:e00405-17. <https://doi.org/10.1128/mSphere.00405-17>.
14. Madhi SA, Venter M, Madhi A, Petersen MK, Klugman KP. 2001. Differing manifestations of respiratory syncytial virus-associated severe lower respiratory tract infections in human immunodeficiency virus type 1-infected and uninfected children. *Pediatr Infect Dis J* 20:164–170. <https://doi.org/10.1097/00006454-200102000-00010>.
15. Madhi SA, Schoub B, Simmank K, Blackburn N, Klugman KP. 2000. Increased burden of respiratory viral associated severe lower respiratory tract infections in children infected with human immunodeficiency virus type-1. *J Pediatr* 137:78–84. <https://doi.org/10.1067/mpd.2000.105350>.
16. Garbino J, Inoubli S, Mossdorf E, Weber R, Tamm M, Soccia P, Aubert J-D, Bridevaux P-O, Tapparel C, Kaiser L. Swiss HIV Cohort Study. 2008. Respiratory viruses in HIV-infected patients with suspected respiratory opportunistic infection. *AIDS* 22:701–705. <https://doi.org/10.1097/QAD.0b013e32824f70ac>.
17. Venter M, Lassauniere R, Kresfelder TL, Westerberg Y, Visser A. 2011. Contribution of common and recently described respiratory viruses to annual hospitalizations in children in South Africa. *J Med Virol* 83:1458–1468. <https://doi.org/10.1002/jmv.22120>.
18. Ogimi C, Englund JA, Bradford MC, Qin X, Boeckh M, Waghmare A. 2019. Characteristics and outcomes of coronavirus infection in children: the role of viral factors and an immunocompromised state. *J Pediatric Infect Dis Soc* 8:21–28. <https://doi.org/10.1093/jpids/pix093>.
19. Nunes MC, Kuschner Z, Rabede Z, Cutland CL, Madimabe R, Kuwanda L, Klugman KP, Adrian PV, Madhi SA. 2014. Polyomaviruses-associated respiratory infections in HIV-infected and HIV-uninfected children. *J Clin Virol* 61:571–578. <https://doi.org/10.1016/j.jcv.2014.10.013>.
20. Madhi SA, Klugman KP, Vaccine Trialist Group. 2004. A role for Streptococcus pneumoniae in virus-associated pneumonia. *Nat Med* 10:811–813. <https://doi.org/10.1038/nm1077>.
21. Madhi SA, Ludewick H, Kuwanda L, van Niekerk N, Cutland C, Little T, Klugman KP. 2006. Pneumococcal coinfection with human metapneumovirus. *J Infect Dis* 193:1236–1243. <https://doi.org/10.1086/503053>.
22. Nunes MC, Kuschner Z, Rabede Z, Madimabe R, Van Niekerk N, Moloi J, Kuwanda L, Rossen JW, Klugman KP, Adrian PV, Madhi SA. 2014. Clinical epidemiology of bocavirus, rhinovirus, two polyomaviruses and four coronaviruses in HIV-infected and HIV-uninfected South African children. *PLoS One* 9:e86448. <https://doi.org/10.1371/journal.pone.0086448>.
23. Klugman KP, Madhi SA, Huebner RE, Kohberger R, Mbelle N, Pierce N, Vaccine Trialists Group. 2003. A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection. *N Engl J Med* 349:1341–1348. <https://doi.org/10.1056/NEJMoa035060>.
24. Golda A, Malek N, Dudek B, Zeglen S, Wojarski J, Ochman M, Kucewicz E, Zembala M, Potempa J, Pyrc K. 2011. Infection with human coronavirus NL63 enhances streptococcal adherence to epithelial cells. *J Gen Virol* 92:1358–1368. <https://doi.org/10.1099/vir.0.028381-0>.
25. Madhi SA, Ramasamy N, Bessellar TG, Saloojee H, Klugman KP. 2002. Lower respiratory tract infections associated with influenza A and B viruses in an area with a high prevalence of pediatric human immunodeficiency type 1 infection. *Pediatr Infect Dis J* 21:291–297. <https://doi.org/10.1097/00006454-200204000-00007>.
26. Simon L, Gauvin F, Amre DK, Saint-Louis P, Lacroix J. 2004. Serum procalcitonin and C-reactive protein levels as markers of bacterial infection: a systematic review and meta-analysis. *Clin Infect Dis* 39:206–217. <https://doi.org/10.1086/421997>.
27. Madhi SA, Heera JR, Kuwanda L, Klugman KP. 2005. Use of procalcitonin and C-reactive protein to evaluate vaccine efficacy against pneumonia. *PLoS Med* 2:e38. <https://doi.org/10.1371/journal.pmed.0020038>.
28. Zheng Z, Peng F, Xu B, Zhao J, Liu H, Peng J, Li Q, Jiang C, Zhou Y, Liu S, Ye C, Zhang P, Xing Y, Guo H, Tang W. 2020. Risk factors of critical & mortal COVID-19 cases: a systematic literature review and meta-analysis. *J Infect* 81:e16–e25. <https://doi.org/10.1016/j.jinf.2020.04.021>.
29. Zhou F, Yu T, Du R, Fan G, Liu Y, Liu Z, Xiang J, Wang Y, Song B, Gu X, Guan L, Wei Y, Li H, Wu X, Xu J, Tu S, Zhang Y, Chen H, Cao B. 2020. Clinical course and risk factors for mortality of adult inpatients with COVID-19 in Wuhan, China: a retrospective cohort study. *Lancet* 395:1054–1062. [https://doi.org/10.1016/S0140-6736\(20\)30566-3](https://doi.org/10.1016/S0140-6736(20)30566-3).
30. Langford BJ, So M, Raybardhan S, Leung V, Westwood D, MacFadden DR, Soucy J-PR, Daneman N. 2020. Bacterial co-infection and secondary infection in patients with COVID-19: a living rapid review and meta-analysis. *Clin Microbiol Infect* 26:1622–1629. <https://doi.org/10.1016/j.cmi.2020.07.016>.
31. Cucchiari D, Pericas JM, Riera J, Gumucio R, Coloma M, Nicolas D, Hospital Clinic 4H Team. 2020. Pneumococcal superinfection in COVID-19 patients: a series of 5 cases. *Med Clin (Barc)* 155:502–505. <https://doi.org/10.1016/j.medcli.2020.05.022>.
32. Heimdal I, Moe N, Krokstad S, Christensen A, Skanke LH, Nordbø SA, Døllner H. 2019. Human coronavirus in hospitalized children with respiratory tract infections: a 9-year population-based study from Norway. *J Infect Dis* 219:1198–1206. <https://doi.org/10.1093/infdis/jiy646>.
33. Zar HJ, Barnett W, Stadler A, Gardner-Lubbe S, Myer L, Nicol MP. 2016. Aetiology of childhood pneumonia in a well vaccinated South African birth cohort: a nested case-control study of the Drakenstein Child Health Study. *Lancet Respir Med* 4:463–472. [https://doi.org/10.1016/S2213-2600\(16\)00096-5](https://doi.org/10.1016/S2213-2600(16)00096-5).
34. Woo PCY, Lau SKP, Lam CSF, Lau CCY, Tsang AKL, Lau JHN, Bai R, Teng JLL, Tsang CCC, Wang M, Zheng B-J, Chan K-H, Yuen K-Y. 2012. Discovery of seven novel mammalian and avian coronaviruses in the genus deltacoronavirus supports bat coronaviruses as the gene source of alphacoronavirus and betacoronavirus and avian coronaviruses as the gene source of gammacoronavirus and deltacoronavirus. *J Virol* 86:3995–4008. <https://doi.org/10.1128/JVI.06540-11>.
35. Lu R, Zhao X, Li J, Niu P, Yang B, Wu H, Wang W, Song H, Huang B, Zhu N, Bi Y, Ma X, Zhan F, Wang L, Hu T, Zhou H, Hu Z, Zhou W, Zhao L, Chen J, Meng Y, Wang J, Lin Y, Yuan J, Xie Z, Ma J, Liu WJ, Wang D, Xu W, Holmes EC, Gao GF, Wu G, Chen W, Shi W, Tan W. 2020. Genomic characterisation and epidemiology of 2019 novel coronavirus: implications for virus origins and receptor binding. *Lancet* 395:565–574. [https://doi.org/10.1016/S0140-6736\(20\)30251-8](https://doi.org/10.1016/S0140-6736(20)30251-8).
36. Severance EG, Bossis I, Dickerson FB, Stallings CR, Origeni AE, Sullens A, Yolken RH, Viscidi RP. 2008. Development of a nucleocapsid-based human coronavirus immunoassay and estimates of individuals exposed to coronavirus in a U.S. metropolitan population. *Clin Vaccine Immunol* 15:1805–1810. <https://doi.org/10.1128/CVI.00124-08>.
37. Braun J, Loyal L, Frentsch M, Wendisch D, Georg P, Kurth F, Hippenstiel S, Dingeldey M, Kruse B, Fauchere F, Baysal E, Mangold M, Henze L, Lauster R, Mall MA, Beyer K, Röhm J, Voigt S, Schmitz J, Miltenyi S, Demuth I, Müller MA, Hocke A, Witzentrath M, Suttrop N, Kern F, Reimer U, Wenschuh H, Drosten C, Corman VM, Giesecke-Thiel C, Sander LE, Thiel A. 2020. SARS-

- CoV-2-reactive T cells in healthy donors and patients with COVID-19. *Nature* 587:270–274. <https://doi.org/10.1038/s41586-020-2598-9>.
38. Mateus J, Grifoni A, Tarke A, Sidney J, Ramirez SI, Dan JM, Burger ZC, Rawlings SA, Smith DM, Phillips E, Mallal S, Lammers M, Rubiro P, Quiambao L, Sutherland A, Yu ED, da Silva Antunes R, Greenbaum J, Frazier A, Markmann AJ, Premkumar L, de Silva A, Peters B, Crotty S, Sette A, Weiskopf D. 2020. Selective and cross-reactive SARS-CoV-2 T cell epitopes in unexposed humans. *Science* 370:89–94. <https://doi.org/10.1126/science.abd3871>.
39. Liguoro I, Pilotto C, Bonanni M, Ferrari ME, Pusiol A, Nocerino A, Vidal E, Cogo P. 2020. SARS-COV-2 infection in children and newborns: a systematic review. *Eur J Pediatr* 179:1029–1046. <https://doi.org/10.1007/s00431-020-03684-7>.
40. Feikin DR, Scott JA, Gessner BD. 2014. Use of vaccines as probes to define disease burden. *Lancet* 383:1762–1770. [https://doi.org/10.1016/S0140-6736\(13\)61682-7](https://doi.org/10.1016/S0140-6736(13)61682-7).
41. Madhi SA, Kuwanda L, Cutland C, Klugman KP. 2005. The impact of a 9-valent pneumococcal conjugate vaccine on the public health burden of pneumonia in HIV-infected and -uninfected children. *Clin Infect Dis* 40:1511–1518. <https://doi.org/10.1086/429828>.

## Epidemiology of respiratory infections, including surveillance studies

Wits VIDA being based at Chris Hani Baragwanath Hospital, which is the third largest hospital in the world, with approximately 3200 beds and about 6760 staff members, was a perfect environment to perform hospital-based surveillance studies. I coordinated the paediatric surveillance for respiratory diseases project. Since testing for respiratory pathogens was not performed as normal patient routine care at the hospital, Wits-VIDA was awarded with different research grants, mostly from the Bill & Melinda Gates Foundation, to collect administrative data on paediatric hospitalizations and to perform polymerase chain reaction (PCR) testing. The information collected under these grants was shared with the hospital and the attending physicians.

Multiple international collaborations resulted from the surveillance study and I was part of key publications in the Lancet family on the global burden of respiratory viral infections (Li et al. 2019; Wang et al. 2021; 2024; Wilkins et al. 2023). The richness of seven years of data collection was particular elucidated in an analysis comparing the incidence of all-cause and pathogen-specific lower respiratory tract infections during the COVID-19 pandemic with the pre-pandemic period (Izu et al. 2023).

During this time, I developed an interest in the biology and epidemiology of Respiratory Syncytial Virus (RSV) and I investigated the molecular heterogeneity of RSV in children in collaboration with AstraZeneca (H. Liu et al. 2020).

Another pathogen that gained particular attention was *Bordetella pertussis* due to large outbreaks in very young infants in different parts of the world, even in countries with high vaccine coverage. Using our surveillance platform, I investigated the best respiratory samples needed for *B. pertussis* diagnosis (Marta C. Nunes, Soofie, et al. 2016) and we described the burden of this pathogen among hospitalized infants in our hospital (Soofie et al. 2016). I also performed a retrospective analysis of respiratory illness samples from longitudinal cohorts of HIV-uninfected and HIV-infected women and their infants to evaluate the burden of severe and less severe pertussis illness and the impact of maternal HIV infection. I reported a high incidence of pertussis PCR positivity in infants younger than two years and in their mothers. Although there was a higher point estimate for pertussis infection among HIV-exposed compared with HIV-unexposed infants, this was not statistically significant and was independent of age and vaccination status. Women living with HIV also had a non-significantly higher incidence compared with HIV-uninfected women (Marta C. Nunes, Downs, et al. 2016). To follow on these results, I investigated how mothers could be protected from pertussis disease. To achieve this, I investigated if pregnant women enrolled



in an influenza vaccine placebo-controlled randomized clinical trial were differentially infected by *B. pertussis*. The results of this analysis suggested that influenza vaccination may have had a protective effect on the rates of *B. pertussis* infection among these women. Although it is unlikely that influenza vaccination would affect the ability to diagnose pertussis, our observation shed light on possible mechanisms in the upper respiratory tract that can lead to the synergy between the two pathogens (Marta C. Nunes, Cutland, and Madhi 2018). The paper describing these results is attached below.

## CORRESPONDENCE



## Influenza Vaccination during Pregnancy and Protection against Pertussis

**TO THE EDITOR:** Data from epidemiologic studies and challenge experiments in animal models have shown that infection with the influenza virus can enhance susceptibility to infection by bacteria such as streptococcus, *Haemophilus influenzae*, and *Staphylococcus aureus*.<sup>1</sup> However, the relationship between influenza virus and atypical bacteria — namely, *Bordetella pertussis* — has not been well characterized.<sup>2</sup>

In a randomized, double-blind, placebo-controlled trial of trivalent inactivated influenza vaccine conducted in South Africa in 2011 and 2012, we found that the vaccine against influenza in pregnant women who were not infected with the human immunodeficiency virus was 50.4% efficacious in preventing polymerase chain reaction (PCR)-confirmed influenza infection from the time of enrollment through 24 weeks post partum.<sup>3</sup> Here, we present the results of retrospective testing by means of PCR for *B. pertussis* infection of the pharyngeal specimens obtained from the women at the time of visits to the study clinic because of respiratory illness.

Archived DNA samples collected independently of clinical presentation were tested by means of real-time PCR for the presence of the multi-copy pertussis insertion sequence (IS) *IS481*.<sup>4</sup> If *IS481* cycle threshold values were less than 40, total nucleic acids were extracted from the corresponding respiratory specimen with the use of a NucliSens easyMag (bioMérieux) machine. The samples were then retested for *IS481* and were tested in a duplex reaction for *hIS1001* and *pIS1001* and in a singleplex reaction for the pertussis toxin subunit S1 (*ptx S1*). The primers and probes used and the interpretation of PCR results have been described previously.<sup>4</sup>

Overall, 2116 women were enrolled in the trial, including 1062 in the influenza vaccine group and 1054 in the placebo group. Recipients were followed by weekly active surveillance for any respiratory illness from the time of enrollment through pregnancy and through 24 weeks post partum. A total of 3583 respiratory specimens were obtained from 1361 women, and 3125 of these specimens (87.2%) were tested for *B. pertussis*. Specimens obtained from 11 vaccine recipients tested positive for pertussis on PCR assay, as compared with specimens obtained from 26 placebo recipients (risk ratio, 0.4; 95% confidence interval [CI], 0.2 to 0.8). Furthermore, in 10 women in the vaccine group and in 16 women in the placebo group, the PCR results were indeterminate for pertussis (Table 1). The overall risk ratio, including the episodes of pertussis with indeterminate PCR results, was 0.5 (95% CI, 0.2 to 0.7).

Thirty-three episodes of pertussis among the women occurred after delivery, and specimens obtained from the infants tested positive for

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**Table 1.** Rate of Detection of *Bordetella pertussis* among Women who Participated in a Randomized, Double-Blind, Placebo-Controlled Trial of Trivalent Inactivated Influenza Vaccine.\*

| Variable  | Influenza Vaccine Group (N=1062) | Placebo Group (N=1054) | Risk Ratio (95% CI) | P Value |
|---|----------------------------------|------------------------|---------------------|---------|
| Recipients — no./total no. (%)  |                                  |                        |                     |         |
| ≥1 specimen obtained†   | 675/1062 (63.6)                  | 686/1054 (65.1)        | 1.0 (0.9–1.0)       | 0.46    |
| ≥1 specimen tested for pertussis by means of PCR†                                       | 635/1062 (59.8)                  | 652/1054 (61.9)        | 1.0 (0.9–1.0)       | 0.33    |
| Respiratory specimens tested for pertussis by means of PCR — no./total no. obtained (%) | 1494/1713 (87.2)                 | 1631/1870 (87.2)       | 1.0 (0.9–1.0)       | 0.99    |
| Cases of pertussis — no./total no. of recipients (%)                                    |                                  |                        |                     |         |
| Positive results on PCR‡  | 11/1062 (1.0)§                   | 26/1054 (2.5)¶         | 0.4 (0.2–0.8)       | 0.012   |
| Indeterminate results on PCR  | 10/1062 (0.9)                    | 16/1054 (1.5)          | 0.6 (0.3–1.4)       | 0.23    |
| Overall   | 21/1062 (2.0)                    | 42/1054 (4.0)          | 0.5 (0.2–0.7)       | 0.007   |

\* CI denotes confidence interval, and PCR polymerase chain reaction.

† Respiratory specimens were obtained only from women who presented with a respiratory illness.

‡ Positive results on PCR indicate a PCR reaction with cycle threshold values for IS481 of less than 35 or greater than or equal to 35 to less than 40 and positive for the gene coding for pertussis toxin subunit S1 (*ptx S1*).

§ One specimen also tested positive for influenza A (H1N1).

¶ Two specimens also tested positive for influenza A (H3N2). Two different specimens obtained 22 days apart from one recipient tested positive for pertussis on PCR, and only the first episode was included.

|| Indeterminate results on PCR indicate a PCR reaction with cycle threshold values for IS481 of greater than or equal to 35 to less than 40 and negative for *ptx S1*.

pertussis on PCR at the same time as the mother on five occasions (three in the vaccine group): within 22 days after the episode of pertussis in the mother on two occasions (both in the placebo group), and between 52 and 73 days after the episode of pertussis in the mother on three occasions (all in the placebo group). There were no instances in which both a mother and her infant had an episode of pertussis and the episode in the mother was detected after the episode in the infant.

Our results suggest that influenza vaccination may have had a protective effect on the rates of *B. pertussis* infection among adult women; however, this is a post hoc observation. We think it is unlikely that influenza vaccination would affect the ability to diagnose pertussis, and therefore this observation warrants further investigation of the possible mechanisms in the upper respiratory tract that can lead to the synergy between the two pathogens.

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1. Peltola VT, McCullers JA. Respiratory viruses predisposing to bacterial infections: role of neuraminidase. *Pediatr Infect Dis J* 2004;23:Suppl:S87-S97.
2. Mina MJ, Burke RM, Klugman KP. Estimating the prevalence of coinfection with influenza virus and the atypical bacteria *Bordetella pertussis*, *Chlamydia pneumoniae*, and *Mycoplasma pneumoniae*. *Eur J Clin Microbiol Infect Dis* 2014;33:1585-9.
3. Madhi SA, Cutland CL, Kuwanda L, et al. Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med* 2014;371:918-31.
4. Nunes MC, Downs S, Jones S, van Niekerk N, Cutland CL, Madhi SA. *Bordetella pertussis* Infection in South African HIV-infected and HIV-uninfected mother-infant dyads: a longitudinal cohort study. *Clin Infect Dis* 2016;63:Suppl 4:S174-S180. DOI: 10.1056/NEJMc1705208

## Maternal immunization, including women living with HIV

To protect infants against infections a powerful strategy is vaccinating their mothers during pregnancy. Maternal vaccination is a very interdisciplinary, fast-evolving area of research.

### Influenza

Under my leadership, we conducted two randomized placebo-controlled trials of maternal influenza vaccination in women living with and without HIV, where we demonstrated the efficacy of vaccinating pregnant women against influenza illness in the infants. These findings were pivotal in informing public health recommendations in South Africa and globally regarding prioritization of pregnant women in low-middle income countries for influenza vaccination. In those trials, despite women living with HIV having lower levels of neutralization, hemagglutination inhibition, and H1-hemagglutinin stalk domain antibodies following vaccination compared with those without HIV (Dhar et al. 2020; Marta C. Nunes, Cutland, et al. 2015; Marta C. Nunes et al. 2018), the overall vaccine efficacy against influenza-associated illness was similar in both groups (57.7% and 50.4%, respectively). Protection in the infants against influenza disease was however only achieved in infants born to HIV-uninfected mothers (Madhi et al. 2014).

I described the kinetics of the transplacentally acquired influenza antibodies in the infants of vaccinated women, and determined that the half-life of these antibodies was 43-45 days in HIV-unexposed infants and 56–65 days in HIV-exposed infants (Marta C. Nunes, Cutland, et al. 2015). Following the infants through the first six months of life I found that maternal influenza vaccination protected the infants against influenza disease only during the first three months of life and not to six months of age, as previously hypothesized. This analysis suggested that the most likely mechanism of protection of the infants is through transplacental transfer of maternal antibodies. Estimating the period that infants can be protected through maternal vaccination has important implications, since there is no influenza vaccine licensed for infants younger than six months old (Marta C. Nunes, Cutland, et al. 2016).

Interestingly, when one of my master's students assessed the persistence of influenza antibodies into the second influenza season in the women; we saw that the majority of women who were vaccinated during pregnancy still had antibody levels above the putative threshold for protection against influenza illness one year after vaccination (Mutsaerts et al. 2016).

Performing secondary analysis in the data collected during these trials I also established that maternal influenza vaccination protected young infants against all-cause lower respiratory tract infection (LRTI) hospitalizations. This vaccine-probe analysis helped to more comprehensively describe the influenza disease burden. This key finding suggested a strong role of bacterial and viral co-infections in LRTI in infants. Since young infants are particularly at heightened risk of LRTI hospitalizations, the introduction of strategies that can decrease the burden of LRTI in the very young children is of global interest (Marta C Nunes et al. 2017).

To explore if the immunogenicity of influenza vaccine in pregnant women living with HIV could be increased and afford protection to the infants I designed another randomized clinical trial using different vaccination regimens. In this study I found that while a regimen of double-dose inactivated influenza vaccine induced slightly greater immunogenicity than did a single-dose regimen in pregnant women living with HIV, immunogenicity in the double-dose group was still lower than historical data from the same setting in pregnant women without HIV (Marta C Nunes et al. 2020).

In the clinical trials of influenza vaccination during pregnancy very few cases of influenza disease required hospitalization. The low incidence of influenza-associated hospitalization within each influenza season poses a challenge to demonstrate the effectiveness of influenza vaccination against severe disease. We then conducted a prospective test-negative study over four consecutive influenza seasons aiming to evaluate the effectiveness of influenza vaccination of pregnant women in reducing the risk of influenza-associated hospitalization in their infants during the first six months of life, and to estimate vaccine effectiveness in pregnant or postpartum women. In this study influenza vaccination during pregnancy had an estimated effectiveness of 65% against influenza-associated hospital admissions in young infants, although efficacy was only demonstrated against influenza viruses considered to be vaccine matches (Marta C Nunes, Walaza, et al. 2022).

### Pertussis

To reduce the susceptibility window period for pertussis between birth and the third dose of the primary vaccination series in infancy, acellular pertussis (aP)-containing vaccines are recommended during pregnancy. Nevertheless, the use of aP-containing vaccines during pregnancy had not been adopted into the public immunization programs of any African country until early 2023, when South Africa introduced this strategy. Before that was, however, necessary to investigate the immune responses of aP-containing vaccines in pregnant women with HIV to understand the relative protection of their infants against pertussis compared with offspring born to women without HIV.

I planned an open label phase IV clinical trial to evaluate the immunogenicity and safety of an adult formulation of tetanus–diphtheria–acellular pertussis vaccine in pregnant women living with HIV compared with HIV-uninfected women. We found that vaccination was safe and immunogenic. Pregnant women living with HIV had, however, attenuated humoral immune responses, which could affect the effectiveness of protecting their infants against pertussis compared with those born to women without HIV (Marta C. Nunes et al. 2023). The infants of these vaccinated mothers are currently still being followed-up until they reach two years of age.

Attached key publications illustrating the different studies I conducted.

ORIGINAL ARTICLE

## Influenza Vaccination of Pregnant Women and Protection of Their Infants

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### ABSTRACT

#### BACKGROUND

There are limited data on the efficacy of vaccination against confirmed influenza in pregnant women with and those without human immunodeficiency virus (HIV) infection and protection of their infants.

#### METHODS

We conducted two double-blind, randomized, placebo-controlled trials of trivalent inactivated influenza vaccine (IIV3) in South Africa during 2011 in pregnant women infected with HIV and during 2011 and 2012 in pregnant women who were not infected. The immunogenicity, safety, and efficacy of IIV3 in pregnant women and their infants were evaluated until 24 weeks after birth. Immune responses were measured with a hemagglutination inhibition (HAI) assay, and influenza was diagnosed by means of reverse-transcriptase–polymerase-chain-reaction (RT-PCR) assays of respiratory samples.

#### RESULTS

The study cohorts included 2116 pregnant women who were not infected with HIV and 194 pregnant women who were infected with HIV. At 1 month after vaccination, seroconversion rates and the proportion of participants with HAI titers of 1:40 or more were higher among IIV3 recipients than among placebo recipients in both cohorts. Newborns of IIV3 recipients also had higher HAI titers than newborns of placebo recipients. The attack rate for RT-PCR–confirmed influenza among both HIV-uninfected placebo recipients and their infants was 3.6%. The attack rates among HIV-uninfected IIV3 recipients and their infants were 1.8% and 1.9%, respectively, and the respective vaccine-efficacy rates were 50.4% (95% confidence interval [CI], 14.5 to 71.2) and 48.8% (95% CI, 11.6 to 70.4). Among HIV-infected women, the attack rate for placebo recipients was 17.0% and the rate for IIV3 recipients was 7.0%; the vaccine-efficacy rate for these IIV3 recipients was 57.7% (95% CI, 0.2 to 82.1).

#### CONCLUSIONS

Influenza vaccine was immunogenic in HIV-uninfected and HIV-infected pregnant women and provided partial protection against confirmed influenza in both groups of women and in infants who were not exposed to HIV. (Funded by the Bill and Melinda Gates Foundation and others; ClinicalTrials.gov numbers, NCT01306669 and NCT01306682.)

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\*A complete list of investigators in the Matflu Trial is available in the Supplementary Appendix, available at NEJM.org.

Drs. Madhi and Nunes contributed equally to this article.

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PREGNANT WOMEN ARE DESIGNATED AS A priority group for seasonal influenza vaccination by the World Health Organization (WHO)<sup>1</sup> because of their heightened susceptibility to severe influenza from the second trimester to the early postpartum period.<sup>2,3</sup> Since pregnancy is associated with immunomodulation, including the attenuation of cell-mediated immune responses,<sup>4</sup> the efficacy of inactivated influenza vaccine (IIV) in pregnant women may differ from its efficacy in healthy nonpregnant women and in men.<sup>5</sup> This difference in vaccine efficacy could be further accentuated in pregnant women infected with the human immunodeficiency virus (HIV), who are at heightened risk for severe influenza illness<sup>6-8</sup> because of HIV-related immunosuppression.<sup>9-14</sup>

Reduced attack rates for all-cause febrile respiratory illness among women vaccinated against influenza during pregnancy were observed during the 1957 Asian influenza pandemic in the United States and in a later randomized, controlled trial in Bangladesh.<sup>15,16</sup> A recent case-control study in the United States also reported that pregnant women with influenza confirmed by means of reverse-transcriptase-polymerase-chain-reaction (RT-PCR) assay (hereafter referred to as confirmed influenza) were 44 to 53% less likely to have been vaccinated with trivalent IIV (IIV3) than controls who did not have influenza.<sup>17</sup> To our knowledge, no published randomized, controlled trial has assessed the efficacy of IIV in preventing confirmed influenza in pregnant women with HIV infection and those without HIV infection.<sup>18,19</sup>

The vaccination of pregnant women may also confer partial protection against confirmed influenza in their infants, as reported in the Bangladeshi trial (in which infants whose mothers had been vaccinated with IIV3 were 63% less likely to have influenza [confirmed by means of enzyme-linked immunosorbent assay]).<sup>16</sup> However, observational studies have had conflicting results with regard to the efficacy of IIV3 vaccination during pregnancy in protecting infants against all-cause respiratory illness.<sup>20-23</sup> The protection of infants 6 months of age or younger against influenza is a public health priority. These infants are at high risk for influenza-associated hospitalization, and their immune responses to IIV vaccination are poor.<sup>24-26</sup> We conducted two studies, one involving pregnant women without

HIV infection and the other involving pregnant women with HIV infection, to evaluate the safety, immunogenicity, and efficacy of IIV3 in these women and in their infants until 24 weeks post partum.

## METHODS

### STUDY DESIGN, OBJECTIVES, AND OVERSIGHT

The two studies were randomized, double-blind, placebo-controlled trials conducted in Soweto, South Africa, where antenatal HIV testing is routine. The enrollment of HIV-uninfected pregnant women was initiated at four antenatal clinics before the onset of the 2011 influenza season (March 3 through August 4) and the 2012 season (March 6 through July 2). Members of the HIV-infected cohort were enrolled at the same facilities (March 3 through June 2, 2011). Eligibility criteria for both cohorts included an age of 18 to 38 years, an estimated gestation of 20 to 36 weeks, and the ability to understand and comply with planned study procedures. (The methods used to determine gestational age and the exclusion criteria are provided in the Supplementary Appendix, which is available with the full text of this article, along with the original and final protocols, at NEJM.org.) All HIV-infected mother-infant dyads in 2011 and a subset of HIV-uninfected dyads were included in a nested immunogenicity study that was performed during each year of the trial. The primary objectives for the cohort of women without HIV infection were to evaluate the efficacy of IIV3 vaccination during pregnancy in protecting their infants against confirmed influenza through 24 weeks of age and to compare seroconversion rates between IIV3 recipients and placebo recipients 1 month after vaccination. Secondary objectives included measuring vaccine efficacy against confirmed influenza in all women until 24 weeks post partum. In the HIV-infected cohort, an additional primary objective was to evaluate the immunogenicity of IIV3 in the women. Secondary objectives for this cohort included measuring vaccine efficacy against confirmed influenza in the women and their infants until 24 weeks post partum. Additional secondary objectives for both cohorts are listed in the Supplementary Appendix.

The studies were approved by the Human Research Ethics Committee of the University of the Witwatersrand and were conducted in accor-



dance with Good Clinical Practice guidelines. Written informed consent was obtained from all participants. All the authors vouch for the fidelity of this report to the protocols and the completeness of the data and analyses. The funders did not participate in the conduct of the study, data collection, analyses of the data, or the writing of the manuscript.

#### RANDOMIZATION AND STUDY TREATMENT

Participants were randomly assigned in a 1:1 ratio to receive IIV3 or placebo. Randomization was performed by the study statistician with the use of computer-generated assignments. With the exception of the statistician and the pharmacist, study personnel were unaware of the group assignments, as were the study participants.

Influenza vaccine (Vaxigrip, lot number G05831 in 2011 and H7221-2 in 2012; Sanofi Pasteur) was purchased by the study team. The vaccine contained 15  $\mu$ g each of A/California/7/2009 (A/[H1N1]pdm09), A/Victoria/210/2009 (A/H3N2), and a B/Brisbane/60/2008–like virus (B/Victoria), as recommended by WHO for the Southern Hemisphere in 2011 and 2012.<sup>27,28</sup> The study pharmacist used a 2-ml syringe to draw 0.5 ml of vaccine for the women receiving IIV3 and 0.5 ml of sterile 0.9% normal saline solution for the women receiving placebo. The two preparations were macroscopically indistinguishable. The vaccines were administered into the deltoid muscle by study staff.

#### SEROLOGIC EFFICACY AND IMMUNOGENICITY

In 2011 and 2012, all mothers and infants in the HIV-infected cohort and a nested group of 180 HIV-uninfected mother–infant dyads were enrolled in an intensive safety and immunogenicity study. The timing of blood-sample collection and processing, the method used for antibody testing (hemagglutination inhibition [HAI]), and the standard criteria used to qualify HAI results as seroconversion and as seroprotective or seronegative titers are provided in the Supplementary Appendix.

#### VACCINE EFFICACY

Active surveillance for influenza-like illness (as defined in the protocols) was conducted through weekly contact with participants (see the Supplementary Appendix for details). Women and infants identified as having influenza-like illness, and those unexpectedly presenting with or hospitalized for any respiratory illness, were tested for influ-

enza virus by means of an RT-PCR assay. The laboratory methods used for sample collection, influenza-virus identification, and genotyping are detailed in the Supplementary Appendix.

#### SAFETY

Women enrolled in the nested immunogenicity subsets were provided with diary cards on which to document possible local and systemic reactions to vaccination for 1 week. Digital thermometers were provided to measure oral temperature in women and axillary temperature in infants after vaccination and during illness. Serious adverse events were recorded and graded throughout the study period with the use of an established system.<sup>29</sup>

#### STATISTICAL ANALYSIS

In the HIV-uninfected cohort, the sample size was based on the primary outcome of vaccine efficacy in the infants. The sample size for the HIV-uninfected cohort was outcome-driven. We aimed at identifying at least 27 cases of confirmed illness caused by influenza virus in infants up to 24 weeks of age in order to detect a 70% reduction in confirmed influenza among the infants, with 80% power. The sample size required for the HIV-infected cohort was 180 participants, which provided 90% power to detect a difference of at least 67% in rates of seroconversion to individual vaccine strains between IIV3 recipients and placebo recipients.

The immunogenicity analyses included comparisons of geometric mean titers between study groups and of the increase in titers from baseline to 1 month after vaccination. We performed a two-sided, two-sample t-test and calculated the corresponding 95% confidence intervals for the titers, using logarithmic transformation for all values. Post-vaccination analyses of immune response were adjusted for baseline HAI titers and between-group differences in baseline characteristics. The proportions of participants in each group who underwent seroconversion and the proportions of participants who had local or systemic reactions were compared by means of chi-square or Fischer's exact tests. Vaccine efficacy was calculated with the use of the formula  $(1-I_v)/I_p$ , where  $I_v$  is the case incidence rate in the vaccinated group and  $I_p$  is the case incidence rate in the placebo group; 95% confidence intervals were calculated and between-group differences were

tested. Estimates of vaccine efficacy were adjusted for differences in maternal age and status with respect to antiretroviral treatment at enrollment, which were prevalent in the HIV-infected cohort. For vaccine efficacy end points, data were censored after the first episode of a specific clinical outcome. Between-group differences in the time to a first episode of confirmed influenza were compared in survival analyses by means of the log-rank test.

The intention-to-treat analyses included maternal outcomes from receipt of vaccine or placebo to 175 days after birth and infant outcomes from birth to 175 days of age. The per-protocol analyses included maternal outcomes that occurred 14 or more days after receipt of vaccine or placebo; per-protocol analyses of outcomes for infants were limited to those born at least 28 days after their mother's vaccination, those whose gestational age at birth was at least 37 weeks, or those who had a birth weight of at least 2500 g. The per-protocol immunogenicity analysis was limited to women from whom a blood sample was obtained 28 to 35 days after vaccination and to infants from whom a blood sample was obtained within 7 days after birth and who had a gestational age of at least 37 weeks at birth or a birth weight of at least 2500 g. An exploratory analysis was performed with the use of extended windows for obtaining blood samples after vaccination (28 to 42 days) and after delivery (up to 14 days).

Study data were collected and managed with the use of Research Electronic Data Capture (REDCap), version 5.9.13.<sup>30</sup> All statistical analyses were conducted with the use of Stata software, version 12.1. All P values were two-sided, and a value of 0.05 or less was considered to indicate statistical significance.

## RESULTS

### BASELINE CHARACTERISTICS OF THE STUDY PARTICIPANTS

We enrolled 2116 black African pregnant women who were not infected with HIV; 1062 were randomly assigned to the group vaccinated with IIV3 and 1054 to the group that received placebo. There were 1026 live infants born to IIV3 recipients and 1023 live infants born to placebo recipients (Fig. S1 in the Supplementary Appendix). The mean maternal age at enrollment was 26.2 years, and the mean gestational age was 26.8 weeks (Table 1).

We enrolled 194 pregnant women who were infected with HIV; 100 were randomly assigned to the group vaccinated with IIV3 and 94 to the placebo group (Fig. S2 in the Supplementary Appendix). The mean maternal age at enrollment was 28.2 years, and the mean gestational age was 27.3 weeks (Table 2). The baseline median CD4+ T-cell count in the HIV-infected women was 393.5 cells per cubic millimeter; 12.6% of the women (24 of 190) had counts of less than 200 cells per cubic millimeter. The median level of plasma HIV-1 RNA was 1067 copies per milliliter; 23.0% of the women (43 of 187) had undetectable levels of HIV-1 RNA. There were 100 live births in the group vaccinated with IIV3 and 88 live births in the placebo group among the 183 HIV-infected women who remained in the study until delivery.

### IMMUNOGENICITY OF THE VACCINE

#### *HIV-Uninfected Cohort*

Of the 376 participants in the immunogenicity subset, 142 IIV3 recipients and 148 placebo recipients were included in the per-protocol analysis of immunogenicity (Fig. S3 and Table S1 in the Supplementary Appendix). Post-vaccination geometric mean titers increased from baseline by a factor of 6 to 10 in IIV3 recipients and were significantly higher for all three vaccine strains, as compared with titers in placebo recipients (Table 3). Seroconversion rates for strains A(H1N1)pdm09, A(H3N2), and B(Victoria) among IIV3 recipients were 72.5%, 64.8%, and 92.3%, respectively; rates among placebo recipients were 8.1%, 2.7%, and 2.0%, respectively ( $P < 0.001$  for all comparisons). A greater proportion of IIV3 recipients had seroprotective HAI titers after vaccination, as compared with placebo recipients (Table 3).

Newborns of IIV3 recipients had higher HAI geometric mean titers for all vaccine strains than did newborns of placebo recipients and were also more likely to have an HAI titer of 1:40 or higher for A(H1N1)pdm09 (81.1% vs. 34.0%), A(H3N2) (60.0% vs. 17.5%), and B(Victoria) (82.1% vs. 43.7%) ( $P < 0.001$  for all comparisons) (Table 3). The ratios of HAI titers in newborns to maternal titers were 0.7 to 1.0 for the three vaccine strains and were similar between study groups, with the exception of higher ratios for B/Victoria in the placebo group (Table 3). Similar findings were observed in the immunogenicity analyses when the window for obtaining blood samples was extended (Tables S2 and S3 in the Supplementary Appendix).

**Table 1. Baseline Characteristics of HIV-Uninfected Pregnant Women, Fetal Outcomes, and Newborn Characteristics.\***

| Characteristic or Outcome  | Overall     | IIV3        | Placebo     |
|--|-------------|-------------|-------------|
| <b>Women</b>   |             |             |             |
| Total no. of women   | 2116        | 1062        | 1054        |
| Mean age — yr  | 26.2±5.3    | 26.4±5.3    | 25.9±5.3    |
| Body-mass index†   |             |             |             |
| Median   | 27.6        | 28.0        | 27.4        |
| Interquartile range  | 24.3–31.9   | 24.6–32.1   | 24.1–31.6   |
| Mean gestational age at enrollment — wk                          | 26.8±4.4    | 26.7±4.4    | 26.9±4.4    |
| <b>Gravidity</b>   |             |             |             |
| Median   | 2.0         | 2.0         | 2.0         |
| Interquartile range  | 0.0–3.0     | 0.0–3.0     | 0.0–3.0     |
| <b>Parity</b>  |             |             |             |
| Median   | 1.0         | 1.0         | 1.0         |
| Interquartile range  | 0.0–1.0     | 0.0–1.0     | 0.0–1.0     |
| <b>Fetuses and newborns</b>                                      |             |             |             |
| Total no. of known outcomes                                      | 2081        | 1044        | 1037        |
| Miscarriage, gestational age <28 wk — no. (%)‡                   | 8 (0.4)     | 3 (0.3)§    | 5 (0.5)     |
| Stillbirth, gestational age ≥28 wk — no. (%)‡                    | 24 (1.2)    | 15 (1.4)    | 9 (0.9)     |
| Live birth — no. (%)   | 2049 (98.5) | 1026 (98.3) | 1023 (98.7) |
| Twin birth — no. (%)   | 110 (5.3)   | 60 (5.7)    | 50 (4.8)    |
| Type of delivery — no. (%)                                       |             |             |             |
| Normal vaginal delivery  | 1393 (66.9) | 700 (67.0)  | 693 (66.8)  |
| Cesarian section   | 656 (31.5)  | 326 (31.2)  | 330 (31.8)  |
| Preterm birth, gestational age <37 wk — no. (%)¶                 | 204 (10.0)  | 108 (10.5)  | 96 (9.4)    |
| Overall birth weight — kg¶  **                                   |             |             |             |
| Median   | 3.1         | 3.1         | 3.1         |
| Range  | 0.5–4.8     | 0.5–4.6     | 0.7–4.8     |
| Birth weight of infants born during influenza season — kg¶  **†† |             |             |             |
| Median   | 3.1         | 3.1         | 3.1         |
| Range  | 0.4–4.8     | 0.6–4.6     | 0.4–4.8     |
| Birth weight of <2500 g — no. (%)¶  **                           | 255 (12.5)  | 133 (13.0)  | 122 (12.0)  |
| Death after delivery — no. (%)¶‡‡                                | 22 (1.1)    | 12 (1.2)    | 10 (1.0)    |
| Admission to neonatal nursery — no. (%)¶                         | 142 (6.9)   | 71 (6.9)    | 71 (6.9)    |

\* Plus-minus values are means ±SD. HIV denotes human immunodeficiency virus, and IIV3 trivalent inactivated influenza vaccine.

† The calculations for body-mass index (the weight in kilograms divided by the square of the height in meters) were based on 796 women in the IIV3 group and 789 women in the placebo group.

‡ Details on miscarriages and stillbirths are provided in Table S9 in the Supplementary Appendix.

§ This number includes one medically assisted termination of pregnancy.

¶ Numbers and percentages are based on live births.

|| Numbers are based on 1024 observations in the IIV3 group and 1021 observations in the placebo group.

\*\* No significant difference in birth weight between the study groups was observed, even when weight was stratified according to sex.

†† Numbers are based on 857 observations in the IIV3 group and 874 observations in the placebo group.

‡‡ Further details on newborn deaths are provided in Table S10 in the Supplementary Appendix.

*HIV-Infected Cohort*

The demographic characteristics of HIV-infected women included in the per-protocol analysis of immunogenicity (Fig. S2 in the Supplementary Appendix) are shown in Table S4 in the Supplementary Appendix. The receipt of IIV3 resulted in increases in geometric mean titers of antibodies to A/(H1N1)pdm09, A/H3N2, and B/Victoria by factors of 2.9, 2.4, and 3.2, respectively, and seroconversion rates of 42.9%, 35.7%, and 40.0%, respectively. Furthermore, as compared with placebo recipients, a higher proportion of IIV3 recipients had seroprotective HAI titers after vaccination (Table S5 in the Supplementary Appendix).

The newborns of IIV3 recipients had higher HAI geometric mean titers than did the newborns of placebo recipients. The ratio of geometric mean HAI titers ranged between 0.7 and 1.4; ratios were higher in the placebo group for A/(H1N1)pdm09 ( $P=0.05$ ) and A/H3N2 ( $P=0.01$ ). In the IIV3 group, the proportion of newborns with an HAI titer of 1:40 or higher was 60.7% for A/(H1N1)pdm09, 42.9% for A/H3N2, and 78.6% for B/Victoria; titers for all three strains were higher than those in the placebo group (Table S5 in the Supplementary Appendix). Similar findings were observed in the immunogenicity analyses when extended windows for obtaining blood samples were used (Tables S6 and S7 in Supplementary Appendix).

**VACCINE EFFICACY***HIV-Uninfected Cohort*

The attack rates for confirmed influenza among placebo recipients and IIV3 recipients were 3.6% and 1.8%, respectively, indicating a vaccine efficacy of 50.4% (95% confidence interval [CI], 14.5 to 71.2) (Table 4 and Fig. 1). The vaccine efficacy was similar when determined according to the vaccine strain (46.1%; 95% CI, 6.4 to 69.0). For the 2 years of the study, the predominant strain of influenza virus circulating among placebo recipients was A/H3N2 (57.9%), for which an exploratory analysis identified a vaccine efficacy of 57.5% (95% CI, 7.6 to 80.4).

The attack rate was lower among infants whose mothers were IIV3 recipients (1.9%) than among those whose mothers were placebo recipients (3.6%), indicating a vaccine efficacy of 48.8% (95% CI, 11.6 to 70.4) (Table 4 and Fig. 1). The predominant strains causing illness in the 38 in-

fants of placebo recipients were A/H3N2 (14 infants [37%]), B/Victoria (11 [29%]), and B/Yamagata (12 [32%]). Among infants with confirmed influenza, the numbers infected with the influenza virus strain carried by their mothers were as follows: 1 of 19 infants in the IIV3 group (5.3%) and 11 of 37 in the placebo group (29.7%,  $P=0.04$ ). The vaccine efficacy estimates in the per-protocol analysis were similar to the estimates in the intention-to-treat analysis for the women and their infants (Table S8 in the Supplementary Appendix).

*HIV-Infected Cohort*

In the cohort of HIV-infected mothers, the attack rate was lower among IIV3 recipients than among placebo recipients (7.0% vs. 17.0%), indicating an adjusted vaccine efficacy of 57.7% (95% CI, 0.2 to 82.1) (Table 4 and Fig. 1). The attack rates were 5.0% and 6.8% among infants of IIV3 recipients and infants of placebo recipients, respectively (vaccine efficacy, 26.7%;  $P=0.60$ ) (Table 4 and Fig. 1). In 6 of 11 infants of HIV-infected mothers with confirmed influenza (55%), including 2 infants whose mothers received IIV3, the mothers were infected with the same strain as their infants. Estimates of vaccine efficacy among HIV-infected women and their infants were similar in the per-protocol and intention-to-treat analyses (Table S8 in the Supplementary Appendix).

**SAFETY**

The details of solicited local and systemic reactions in the HIV-uninfected cohort and the HIV-infected cohort are shown in Tables S9 and S10 in the Supplementary Appendix. Injection-site reactions (mainly mild to moderate) were more frequent among IIV3 recipients than among placebo recipients in both cohorts, but there were no other significant differences in solicited reactions between the two study groups in either cohort. Data on serious adverse events in both cohorts, including infant and maternal deaths and hospitalizations, are shown in Tables S11 to S23 in the Supplementary Appendix. There were no significant between-group differences with regard to rates of miscarriage, stillbirth, or premature birth or birth weight in the HIV-uninfected cohort (Table 1, and Table S11 in the Supplementary Appendix) and in the HIV-infected cohort (Table 2). The findings were similar in an analy-

| Characteristic or Outcome   | Overall       | IIV3           | Placebo     |
|---|---------------|----------------|-------------|
| <b>Women</b>  |               |                |             |
| Total no. of women  | 194           | 100            | 94          |
| Mean age — yr   | 28.2±5.1      | 27.2±4.9†      | 29.2±5.1†   |
| Mean body-mass index‡   | 28.7±5.3      | 29.2±5.0       | 28.3±5.7    |
| Mean gestational age at enrollment — wk                                 | 27.3±3.8      | 27.6±3.9       | 26.9±3.7    |
| <b>Gravidity</b>  |               |                |             |
| Median  | 2.0           | 2.0            | 2.0         |
| Interquartile range   | 2.0–3.0       | 2.0–3.0        | 2.0–3.0     |
| <b>Parity</b>   |               |                |             |
| Median  | 1.0           | 1.0            | 1.0         |
| Interquartile range   | 1.0–2.0       | 1.0–2.0        | 1.0–2.0     |
| <b>HAART — no. (%)</b>  |               |                |             |
| At enrollment   | 60 (30.9)     | 22 (22.0)†     | 38 (40.4)†  |
| At delivery   | 77 (39.7)     | 33 (33.0)      | 44 (46.8)   |
| <b>ART for prevention of mother-to-child HIV transmission — no. (%)</b> |               |                |             |
| At enrollment   | 86 (44.3)     | 54 (54.0)†     | 32 (34.0)†  |
| At delivery   | 87 (44.9)     | 52 (52.0)§     | 35 (37.2)§  |
| <b>CD4+ T-lymphocyte count — cells/mm<sup>3</sup></b>                   |               |                |             |
| <b>At enrollment¶</b>   |               |                |             |
| Median  | 393.5         | 410.0          | 379.0       |
| Interquartile range   | 271.0–557.0   | 284.0–572.0    | 245.0–550.0 |
| <b>At post-vaccination visit  </b>                                      |               |                |             |
| Median  | 399.5         | 371.0          | 409.0       |
| Interquartile range   | 265.0–499.0   | 255.0–499.0    | 274.0–500.0 |
| <b>At delivery**</b>  |               |                |             |
| Median  | 446.5         | 441.0          | 508.0       |
| Interquartile range   | 330.0–599.0   | 303.0–549.0    | 346.0–673.0 |
| <b>HIV-1 viral load — copies/ml</b>                                     |               |                |             |
| <b>At enrollment††</b>  |               |                |             |
| Median  | 1067.0        | 1679.0         | 399.0       |
| Interquartile range   | 61.0–13,923.0 | 118.5–15,906.5 | 39.0–9990.0 |
| <b>At post-vaccination visit‡‡</b>                                      |               |                |             |
| Median  | 620.0         | 812.0§         | 292.5§      |
| Interquartile range   | 39.0–8278.0   | 130.0–13,221.0 | 39.0–3056.0 |
| <b>At delivery§§</b>  |               |                |             |
| Median  | 265.0         | 222.0          | 356.0       |
| Interquartile range   | 54.0–2040.0   | 64.0–2262.0    | 39.0–1880.0 |
| <b>Fetuses and newborns</b>   |               |                |             |
| Total no. of live-born infants  | 188           | 100            | 88          |
| Twin birth — no. (%)  | 10 (5.3)      | 6 (6.0)        | 4 (4.5)     |
| <b>Type of delivery — no. (%)</b>                                       |               |                |             |
| Normal vaginal  | 111 (59.0)    | 58 (58.0)      | 53 (60.2)   |
| Cesarian section  | 77 (41.0)     | 42 (42.0)      | 35 (39.8)   |
| Preterm birth, gestational age <37 wk — no. (%)                         | 26 (13.8)     | 13 (13.0)      | 13 (14.8)   |

**Table 2. (Continued.)**

| Characteristic or Outcome                                    | Overall   | IIV3      | Placebo   |
|--|-----------|-----------|-----------|
| Overall birth weight — kg ¶¶                                 |           |           |           |
| Median   | 3.0       | 2.9       | 3.0       |
| Range  | 0.8–4.3   | 0.8–4.3   | 1.9–4.0   |
| Birth weight of infants born during influenza season — kg ¶¶ |           |           |           |
| Median   | 3.0       | 3.0       | 3.0       |
| Range  | 0.8–4.3   | 0.8–4.3   | 1.9–4.1   |
| Birth weight of <2500 g — no. (%) ¶¶                         | 29 (15.6) | 14 (14.1) | 15 (17.2) |
| Death after delivery — no. (%) ***                           | 4 (2.1)   | 2 (2.0)   | 2 (2.3)   |
| Admission to neonatal nursery — no. (%)                      | 9 (4.8)   | 6 (6.0)   | 3 (3.4)   |

\* Plus-minus values are means ± SD. ART denotes antiretroviral therapy, and HAART highly active antiretroviral therapy.  
† P<0.01.  
‡ Data are based on 81 observations in the IIV3 group and 74 observations in the placebo group.  
§ P<0.05.  
¶ Numbers are based on 97 observations in the IIV3 group and 93 observations in the placebo group.  
¶ Numbers are based on 78 observations in the IIV3 group and 68 observations in the placebo group.  
\*\* Numbers are based on 77 observations in the IIV3 group and 69 observations in the placebo group.  
†† Numbers are based on 96 observations in the IIV3 group and 91 observations in the placebo group.  
‡‡ Numbers are based on 77 observations in the IIV3 group and 64 observations in the placebo group.  
§§ Numbers are based on 72 observations in the IIV3 group and 65 observations in the placebo group.  
¶¶ Numbers and percentages are based on 99 observations in the IIV3 group and 87 observations in the placebo group.  
|| Numbers are based on 88 observations in the IIV3 group and 80 observations in the placebo group.  
\*\*\* Further details on newborn deaths are provided in Table S17 in the Supplementary Appendix.

sis stratified according to sex and an analysis limited to births that occurred during the influenza season.

DISCUSSION

The trials of IIV3 efficacy in HIV-infected and HIV-uninfected pregnant women showed a reduction in confirmed cases of influenza in both cohorts. Furthermore, we observed protection against confirmed influenza among infants of IIV3 recipients who were not exposed to HIV, with a similar trend observed in infants exposed to HIV. IIV3 was found to be safe, and the vaccine was immunogenic in HIV-uninfected women and HIV-infected women, supporting the current WHO recommendations for influenza vaccination during pregnancy.<sup>19</sup>

The vaccine efficacy against confirmed influenza in infants who were not exposed to HIV (45.6%; 95% CI, 2.4 to 69.7) was similar to that reported in Bangladesh (63%; 95% CI, 5 to 85).<sup>16</sup> However, direct comparisons with the results of the Bangladesh study are limited because of differences in the assays used for viral detection. In addition, influenza virus circulation in Bangla-

desh is prolonged and perennial, limiting the generalizability of the Bangladesh study to temperate regions with more discrete influenza seasons, such as South Africa.<sup>16</sup> The effectiveness of IIV3 vaccination in protecting the infants of pregnant women not infected with HIV is further corroborated by case-control studies in the United States among American Indians (in which most cases were diagnosed through serologic testing) and by a study of influenza-associated hospitalization in a large urban hospital (in which the diagnosis was confirmed through fluorescent antibody testing).<sup>20,22</sup>

It has been proposed that higher HAI titers may be required to provide protection against influenza in children; in adults, an HAI titer of 1:40 or higher is associated with 50% protection.<sup>31,32,33</sup> Nevertheless, in our study the proportion of HIV-unexposed newborns in the IIV3 group with HAI titers of 1:40 or higher (60 to 82%) suggests that this threshold may be associated with approximately 50% protection against confirmed influenza in such infants. Further studies and analyses are warranted to establish a serocorrelate for the protection of HAI antibodies acquired through the placenta. Another way in which the

vaccination of pregnant women may protect their infants against influenza involves prevention of the transmission of the virus to the infant by reducing the mother's risk of influenza illness. This is corroborated in part by our study, in which concurrent confirmed influenza infection by homotypic strains occurred more frequently in HIV-unexposed infants of placebo recipients than in HIV-unexposed infants of IIV3 recipients; infections with homotypic strains also occurred concurrently in 54.5% of the mothers of HIV-exposed infants with confirmed influenza illness.

Unlike the study in Bangladesh, in which rates of clinical febrile respiratory illness were reduced by 29% and 36% in infants and mothers, respectively,<sup>16</sup> our study did not show any reduction in the less specific outcomes of clinical influenza-like illness or respiratory illness in either infants or mothers. The use of pneumococcal polysaccharide vaccine as the comparator for the control group in the Bangladeshi study, as well as the use of different case definitions, may have contributed to the discrepant findings in the two studies. Observational studies in the United States have also shown that maternal influenza vaccination was not effective in protecting infants against all-cause respiratory illness, indicating the lack of specificity of such end points for the evaluation of influenza vaccine efficacy in infants.<sup>21,23</sup>

The vaccine efficacy against confirmed influenza among HIV-uninfected women in our study (per-protocol analysis, 54.4%; 95% CI, 19.5 to 74.2) was similar to that reported in an observational study of vaccination against monovalent A/(H1N1)pdm09 in pregnant Norwegian women (70%; 95% CI, 66 to 75)<sup>33</sup> and in a more recent case-control study in the United States (44 to 53%).<sup>17</sup> The vaccine efficacy against confirmed influenza in HIV-infected pregnant women in our study (per-protocol analysis, 70.6%) was similar to that reported in a trial of IIV3 in South African HIV-infected men and women during the 2008 influenza epidemic (75.5%).<sup>34</sup> The efficacy of the vaccine in our study was also similar to that reported in a meta-analysis of HIV-infected adults in high-income countries (66%; 95% CI, 36 to 82).<sup>35</sup> Notably, the attack rate among HIV-infected placebo recipients in our study (17.0%) was greater than that observed among HIV-uninfected placebo recipients (3.6%), a finding that highlights the greater susceptibility of HIV-infected persons

to influenza, even when the HIV infection is managed with antiretroviral treatment.

Extrapolation of the potential effect of influenza vaccination in pregnancy solely on the basis of HAI immune responses may be affected by pregnancy-inducing immune tolerance, which could influence vaccine effectiveness.<sup>4,5,36</sup> The availability of immunogenicity and efficacy data in our nested immunogenicity study helped to address this question. The proportions of HIV-uninfected IIV3 recipients with seroprotective HAI titers for A/(H1N1)pdm09, A/H3N2, and B/Victoria were 93.3%, 78.0%, and 96.0%, respectively, with corresponding overall vaccine efficacy against confirmed influenza of 54.4%. These findings suggest that the threshold of 1:40 or higher was predictive of an anticipated vaccine efficacy of 50%, as proposed for healthy adults.<sup>31</sup> Theoretically, the rate of naturally acquired immunity among placebo recipients (20 to 45%) could have decreased the true efficacy of the vaccine, a consideration that would not be required in an immunologically naive placebo group of women not infected with influenza.

Among IIV3 recipients in the HIV-infected cohort, HAI titers of 1:40 or higher were observed for A/H3N2 and A/(H1N1)pdm09 (48.6% and 68.6%, respectively). Consequently, the HAI titer of 1:40 or higher, as a relative serocorrelate of protection, would have paradoxically underestimated vaccine efficacy against confirmed influenza among the HIV-infected women (reported as 70.6%). This observation is consistent with findings from our previous study involving HIV-infected adults.<sup>34</sup> Among the HIV-infected cohort, IIV3 did not affect the plasma HIV-1 viral load, minimizing concern about the possibility of an increased risk of vertical transmission of HIV in vaccinees.<sup>37,38</sup>

One of the limitations of our study is the fact that it was conducted at a single center; the generalizability of the findings needs to be corroborated. Furthermore, the timing of enrollment was contingent on the commercial availability of IIV3. Although we initiated enrollment within 1 week after vaccine availability, vaccination extended into the influenza season, with some births occurring after the influenza season. The difference in duration of exposure to the influenza virus between the women and their infants limited our ability to make a direct comparison of the attack

**Table 3. Immune Responses to IIV3 Vaccine in HIV-Uninfected Pregnant Women and Transplacental Transfer of Antibodies to Newborns.\***

| Measure  | A(H1N1)pdm09            |                        |          | A/H3N2                  |                        |          | B/Victoria              |                        |          |
|--|-------------------------|------------------------|----------|-------------------------|------------------------|----------|-------------------------|------------------------|----------|
|  | IIV3                    | Placebo                | P Value† | IIV3                    | Placebo                | P Value† | IIV3                    | Placebo                | P Value† |
| <b>Mothers</b>   |                         |                        |          |                         |                        |          |                         |                        |          |
| Total no. of mothers                                     | 142                     | 148                    |          | 142                     | 148                    |          | 142                     | 148                    |          |
| Geometric mean HAI titer (95% CI)                        |                         |                        |          |                         |                        |          |                         |                        |          |
| Before vaccination                                       | 30.7 (25.5–37.0)        | 27.2 (21.8–34.0)       | 0.41     | 14.6 (12.4–17.0)        | 14.0 (11.8–16.6)       | 0.74     | 17.6 (15.6–19.9)        | 15.4 (13.4–17.7)       | 0.15     |
| After vaccination  | 213.4 (175.7–259.2)     | 29.1 (23.0–36.8)       | <0.001   | 87.8 (70.5–109.2)       | 16.0 (13.3–19.1)       | <0.001   | 175.5 (148.7–207.3)     | 16.9 (14.6–19.6)       | <0.001   |
| P value‡   | <0.001                  | 0.30                   |          | <0.001                  | 0.005                  |          | <0.001                  | 0.05                   |          |
| Factor increase in geometric mean HAI titer (95% CI)     | 6.9 (5.6–8.6)           | 1.1 (0.9–1.2)          | <0.001   | 6.0 (4.9–7.4)           | 1.1 (1.0–1.2)          | <0.001   | 10.0 (8.4–11.8)         | 1.1 (1.0–1.2)          | <0.001   |
| HAI titer ≥1:40 — no. (%) (95% CI)                       |                         |                        |          |                         |                        |          |                         |                        |          |
| Before vaccination                                       | 73 (51.4); (42.9–59.9)  | 66 (44.6); (36.4–53.0) | 0.73     | 33 (23.2); (16.6–31.1)  | 37 (25.0); (18.3–32.8) | 0.25     | 29 (20.4); (14.1–27.7)  | 30 (20.3); (14.1–28.0) | 0.97     |
| After vaccination  | 133 (93.7); (88.3–97.1) | 71 (47.8); (40.4–57.0) | <0.001   | 112 (78.9); (71.2–85.3) | 40 (27.0); (20.1–35.0) | <0.001   | 138 (97.2); (92.9–99.2) | 43 (29.1); (21.9–37.1) | <0.001   |
| P value‡   | 0.006                   | 0.37                   |          | 0.001                   | 0.43                   |          | <0.001                  | <0.001                 |          |
| Seroconversion 1 mo after vaccination — no. (%) (95% CI) | 103 (72.5); (64.4–79.7) | 12 (8.1); (4.3–13.7)   | <0.001   | 92 (64.8); (56.3–72.6)  | 4 (2.7); (0.7–6.8)     | <0.001   | 131 (92.3); (86.6–96.1) | 3 (2.0); (0.4–5.8)     | <0.001   |
| <b>Newborns</b>  |                         |                        |          |                         |                        |          |                         |                        |          |
| Total no. of newborns                                    | 95                      | 103                    |          | 95                      | 103                    |          | 95                      | 103                    |          |
| Geometric mean HAI titer at ≤7 days of age (95% CI)      | 93.3 (71.1–122.4)       | 17.2 (13.8–22.7)       | <0.001   | 41.8 (31.6–55.3)        | 12.7 (10.6–15.1)       | <0.001   | 80.6 (64.9–100.1)       | 21.4 (17.8–25.7)       | <0.001   |
| Ratio of newborn to maternal HAI titer (95% CI)§         | 0.7 (0.6–0.8)           | 0.7 (0.6–0.8)          | 0.78     | 0.7 (0.6–0.9)           | 0.7 (0.6–0.8)          | 0.49     | 0.8 (0.7–0.9)           | 1.0 (0.9–1.2)          | 0.04     |
| HAI titer ≥1:40 at <7 days of age — no. (%) (95% CI)¶    | 77 (81.1); (71.7–88.4)  | 35 (34.0); (24.9–44.0) | <0.001   | 57 (60.0); (49.4–69.9)  | 18 (17.5); (10.7–26.2) | <0.001   | 78 (82.1); (72.9–89.2)  | 45 (43.7); (33.9–53.8) | <0.001   |

\* HAI denotes hemagglutination inhibition.  
 † These P values are for the comparison of the IIV3 and placebo groups.  
 ‡ These P values are for the comparison of values within a group before and after vaccination.  
 § The ratio of the newborn HAI titer to the maternal HAI titer was based on 93 observations in the IIV3 group and 102 observations in the placebo group.  
 ¶ P values were adjusted for the mean number of days from maternal vaccination to birth.



**Table 4. Efficacy of HIV3 Vaccination in Mothers and Infants until 24 Weeks after Birth, Intention-to-Treat Population.\***

| Efficacy End Point                             | HIV-Uninfected Cohort         |                               |                     | HIV-Infected Cohort          |                               |                       |
|--|-------------------------------|-------------------------------|---------------------|------------------------------|-------------------------------|-----------------------|
|  | IV3<br>(N = 1026)             | Placebo<br>(N = 1023)         | Vaccine<br>Efficacy | IV3<br>(N = 100)             | Placebo<br>(N = 88)           | Vaccine<br>Efficacy   |
| <b>Infants</b>                                 |                               |                               |                     |                              |                               |                       |
| RT-PCR-confirmed influenza — no. (%); (95% CI) |                               |                               |                     |                              |                               |                       |
| With inclusion of B/Yamagata                   | 19 (1.9);<br>(1.1 to 2.9)†    | 37 (3.6);<br>(2.6 to 5.0)†‡   | 48.8 (11.6 to 70.4) | 5 (5.0);<br>(1.6 to 11.3)§   | 6 (6.8);<br>(2.5 to 14.3)§    | 26.7 (-132.0 to 76.8) |
| With exclusion of B/Yamagata                   | 13 (1.3);<br>(0.7 to 2.2)     | 25 (2.4);<br>(1.6 to 3.6)     | 48.2 (-0.8 to 73.3) | 4 (4.0);<br>(1.1 to 9.9)     | 3 (3.4);<br>(0.7 to 9.6)      | 14.8 (-270.4 to 80.4) |
| Influenza-like illness — no. (%); (95% CI)     | 595 (58.0);<br>(54.9 to 61.0) | 584 (57.1);<br>(54.0 to 60.1) | -1.6 (-9.4 to 5.7)  | 66 (66.0);<br>(55.8 to 75.2) | 57 (64.8);<br>(53.9 to 74.7)  | -1.9 (-25.5 to 17.3)  |
| Any respiratory illness — no. (%); (95% CI)    | 706 (68.8);<br>(65.9 to 71.6) | 697 (68.1);<br>(65.2 to 71.0) | -1.0 (-7.1 to 4.8)  | 76 (76.0);<br>(66.4 to 84.0) | 70 (79.5);<br>(69.6 to 87.4)  | 4.5 (-11.3 to 18.0)   |
| <b>Women</b>                                   |                               |                               |                     |                              |                               |                       |
| RT-PCR confirmed influenza — no. (%)           |                               |                               |                     |                              |                               |                       |
| With inclusion of B/Yamagata                   | 19 (1.8);<br>(1.1 to 2.8)¶    | 38 (3.6);<br>(2.6 to 4.9)¶    | 50.4 (14.5 to 71.2) | 7 (7.0);<br>(2.9 to 13.9)¶   | 16 (17.0);<br>(10.1 to 26.2)¶ | 57.7 (0.2 to 82.1)    |
| With exclusion of B/Yamagata                   | 19 (1.8);<br>(1.1 to 2.8)     | 35 (3.3);<br>(2.3 to 4.6)     | 46.1 (6.4 to 69.0)  | 7 (7.0);<br>(2.9 to 13.9)    | 13 (13.8);<br>7.6 to 22.5     | 48.2 (-27.0 to 78.8)  |
| Influenza-like illness — no. (%)               | 175 (16.5);<br>(14.3 to 18.8) | 181 (17.2);<br>(14.9 to 19.6) | 4.0 (-16.0 to 20.6) | 24 (24.0);<br>(16.0 to 33.6) | 27 (28.7);<br>(19.9 to 39.0)  | -0.07 (-63.7 to 38.8) |
| Any respiratory illness — no. (%)              | 674 (63.5);<br>(60.5 to 66.4) | 687 (65.2);<br>(62.2 to 68.1) | 2.6 (-3.7 to 8.6)   | 72 (72.0);<br>(62.1 to 80.5) | 73 (77.7);<br>(67.9 to 85.6)  | 5.2 (-12.2 to 19.8)   |

\* Vaccine efficacy in HIV-infected women was adjusted for maternal age and status with respect to antiretroviral treatment at enrollment. RT-PCR denotes reverse-transcriptase–polymerase-chain-reaction.

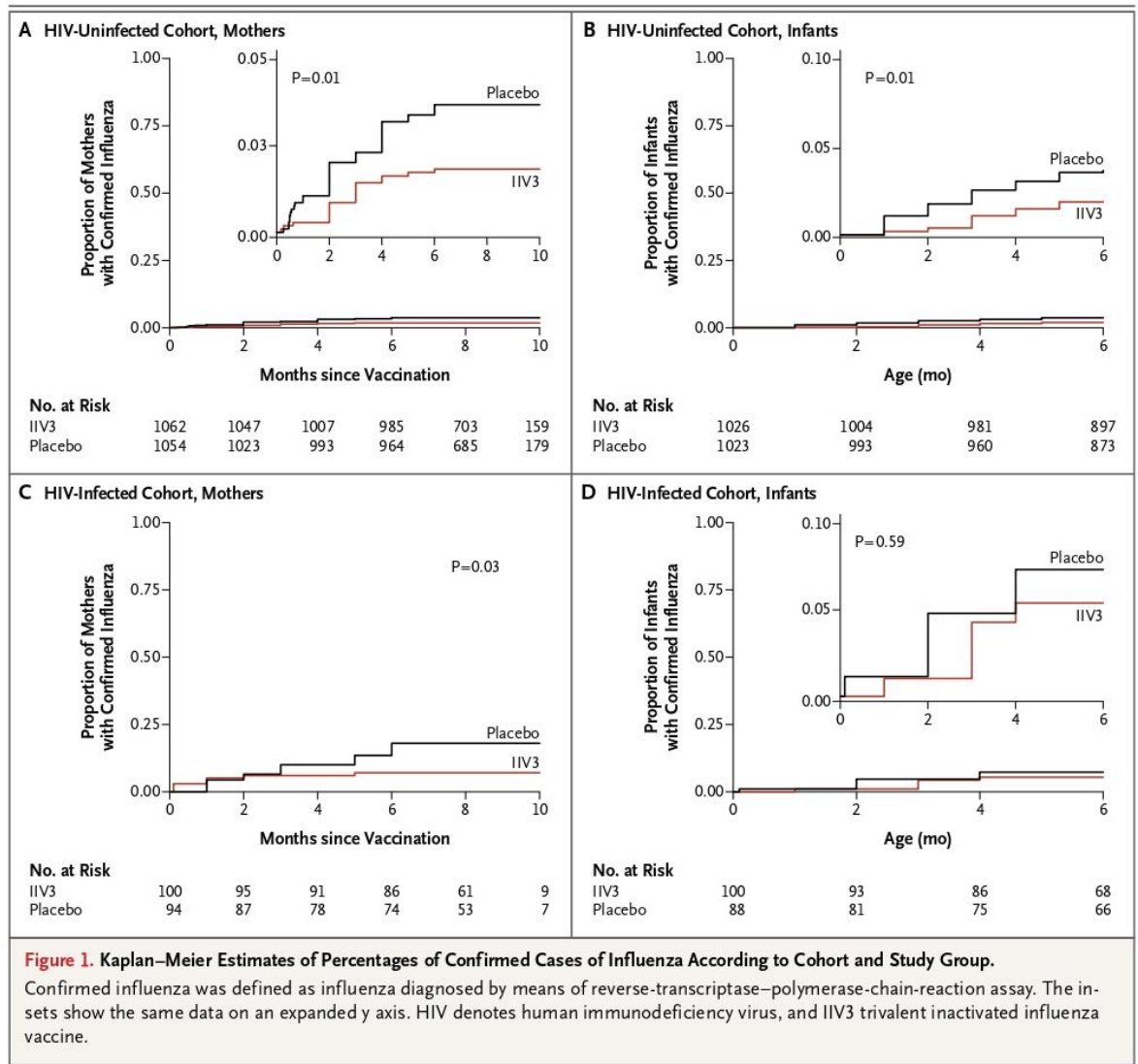
† Among HIV-unexposed infants of IV3 recipients and placebo recipients; there were 0 and 1 cases of infection with A/(H1N1)pdm09, 7 and 14 cases of infection with A/H3N2, 6 and 11 cases of infection with B/Victoria, and 6 and 12 cases of infection with B/Yamagata, respectively.

‡ One baby born to a placebo recipient had a single influenza episode during which both A/(H1N1)pdm09 and A/H3N2 were detected.

§ Among HIV-exposed infants of IV3 recipients and placebo recipients, infection with A/(H1N1)pdm09 occurred in 2 and 0 infants, respectively, infection with A/H3N2 occurred in 2 infants in each group, infection with B/Victoria occurred in 0 and 1 infants, respectively, and infection with B/Yamagata occurred in 1 and 3 infants, respectively.

¶ Among HIV-uninfected IV3 recipients and placebo recipients, there were 5 and 6 cases of influenza virus strain A/(H1N1)pdm09, 10 and 22 cases of A/H3N2, 4 and 7 cases of B/Victoria, and 0 and 3 cases of B/Yamagata, respectively.

|| Among HIV-infected IV3 recipients and placebo recipients, there were 6 and 8 cases of influenza virus strain A/(H1N1)pdm09, 1 and 4 cases of A/H3N2, 0 and 1 case of B/Victoria, and 0 and 3 cases of B/Yamagata, respectively.



rates between them and between these infants and those in the Bangladeshi study, for whom exposure was probably more prolonged.<sup>16,39</sup> Another limitation of our study is the fact that the evaluation of vaccine efficacy among HIV-infected women was a secondary objective; nevertheless, its efficacy in these women was indicated by the high attack rate in the placebo group. However, the study lacked power to evaluate vaccine efficacy among the infants of HIV-infected women, for whom the point estimate was similar to that for HIV-uninfected infants in the per-

protocol analysis. Although 23% of the HIV-infected and uninfected immunogenicity cohorts were outside the protocol-defined windows for obtaining post-vaccination blood samples, the results were similar to those included in the exploratory immunogenicity analysis with an expanded window. An inadvertent occurrence in the study was the difference in HIV disease characteristics between the placebo group and the IIV3 group, including the fact that placebo recipients were more likely to be receiving highly active antiretroviral therapy. However, this factor would

have biased the results in the direction of a reduced risk of influenza in the placebo group.<sup>7</sup> In addition, in HIV-infected women, the lower HAI titers among placebo recipients as compared with IIV3 recipients could have been due to differences in HIV disease stage between the groups.

In conclusion, these data show that IIV3 vaccination in pregnant HIV-uninfected and HIV-infected African women was immunogenic and provided protection against confirmed influenza. The vaccination was also effective in HIV-unexposed infants up until 24 weeks after birth.

The contents of this report are solely the responsibility of the authors and do not necessarily represent the official views of their institutions or organizations or of the study sponsors.

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#### APPENDIX

From the Medical Research Council, Respiratory and Meningeal Pathogens Research Unit (S.A.M., C.L.C., L.K., A.H., S.J., P.V.A., N.N., K.P.K., M.C.N.), the Department of Science and Technology–National Research Foundation, Vaccine-Preventable Diseases (S.A.M., C.L.C., L.K., A.H., S.J., P.V.A., N.N., M.C.N.), and the Perinatal HIV Research Unit (A.V.), University of the Witwatersrand, the National Institute for Communicable Diseases, the National Health Laboratory Service, Centre for Vaccines and Immunology (S.A.M., E.T., M.V.), Johannesburg, and the Department of Medical Virology, University of Pretoria, Pretoria (M.V.) — all in South Africa; the School of Medicine and Children's Hospital, University of Colorado (A.W.), the Department of Pediatrics, Medicine and Pathology, University of Colorado School of Medicine (E.A.F.S.), and the Center for Global Health, Department of Epidemiology, Colorado School of Public Health (E.A.F.S.) — all in Aurora, Colorado; the Department of Medicine and Department of Global Health, University of Washington (J.R.O.), and the Vaccine Access and Delivery Global Program, PATH (J.R.O., K.M.N.) — both in Seattle; and the Hubert Department of Global Health, Rollins School of Public Health, and the Division of Infectious Diseases, School of Medicine, Emory University, Atlanta (K.P.K.).

#### REFERENCES

- Meeting of the Strategic Advisory Group of Experts on Immunization, April 2012 — conclusions and recommendations. *Wkly Epidemiol Rec* 2012;87:201-16.
- Neuzil KM, Reed GW, Mitchel EF, Simonsen L, Griffin MR. Impact of influenza on acute cardiopulmonary hospitalizations in pregnant women. *Am J Epidemiol* 1998;148:1094-102.
- Louie JK, Acosta M, Jamieson DJ, et al. Severe 2009 H1N1 influenza in pregnant and postpartum women in California. *N Engl J Med* 2010;362:27-35.
- Jamieson DJ, Theiler RN, Rasmussen SA. Emerging infections and pregnancy. *Emerg Infect Dis* 2006;12:1638-43.
- Schlaudecker EP, McNeal MM, Dodd CN, Ranz JB, Steinhoff MC. Pregnancy modifies the antibody response to trivalent influenza immunization. *J Infect Dis* 2012;206:1670-3.
- Cohen C, Simonsen L, Sample J, et al. Influenza-related mortality among adults aged 25-54 years with AIDS in South Africa and the United States of America. *Clin Infect Dis* 2012;55:996-1003.
- Neuzil KM, Coffey CS, Mitchel EF Jr, Griffin MR. Cardiopulmonary hospitalizations during influenza season in adults and adolescents with advanced HIV infection. *J Acquir Immune Defic Syndr* 2003; 34:304-7.
- Archer B, Cohen C, Naidoo D, et al. Interim report on pandemic H1N1 influenza virus infections in South Africa, April to October 2009: epidemiology and factors associated with fatal cases. *Euro Surveill* 2009;14:19369.
- Staprans SI, Hamilton BL, Follansbee SE, et al. Activation of virus replication after vaccination of HIV-1-infected individuals. *J Exp Med* 1995;182:1727-37.
- Nelson KE, Clements ML, Miotti P, Cohn S, Polk BF. The influence of human immunodeficiency virus (HIV) infection on antibody responses to influenza vaccines. *Ann Intern Med* 1988;109:383-8.
- Miotti PG, Nelson KE, Dallabetta GA, Farzadegan H, Margolick J, Clements ML. The influence of HIV infection on antibody responses to a two-dose regimen of influenza vaccine. *JAMA* 1989;262:779-83.
- Fuller JD, Craven DE, Steger KA, Cox N, Heeren TC, Chernoff D. Influenza vaccination of human immunodeficiency virus (HIV)-infected adults: impact on plasma levels of HIV type 1 RNA and determinants of antibody response. *Clin Infect Dis* 1999;28:541-7.
- Benne CA, Kroon FP, Harmsen M, Tavares L, Kraaijeveld CA, De Jong JC. Comparison of neutralizing and hemagglutination-inhibiting antibody responses to influenza A virus vaccination of human immunodeficiency virus-infected individuals. *Clin Diagn Lab Immunol* 1998;5: 114-7.
- Kroon FP, van Dissel JT, de Jong JC, Zwinderman K, van Furth R. Antibody response after influenza vaccination in HIV-infected individuals: a consecutive 3-year study. *Vaccine* 2000;18:3040-9.
- Hulka JF. Effectiveness of polyvalent influenza vaccine in pregnancy: report of a controlled study during an outbreak of Asian influenza. *Obstet Gynecol* 1964;23: 830-7.
- Zaman K, Roy E, Arifeen SE, et al. Effectiveness of maternal influenza immu-

- nization in mothers and infants. *N Engl J Med* 2008;359:1555-64.
17. Thompson MG, Li DK, Shifflett P, et al. Effectiveness of seasonal trivalent influenza vaccine for preventing influenza virus illness among pregnant women: a population-based case-control study during the 2010-2011 and 2011-2012 influenza seasons. *Clin Infect Dis* 2014;58:449-57.
  18. Ortiz JR, Englund JA, Neuzil KM. Influenza vaccine for pregnant women in resource-constrained countries: a review of the evidence to inform policy decisions. *Vaccine* 2011;29:4439-52.
  19. Vaccines against influenza WHO position paper — November 2012. *Wkly Epidemiol Rec* 2012;87:461-76.
  20. Benowitz I, Esposito DB, Gracey KD, Shapiro ED, Vázquez M. Influenza vaccine given to pregnant women reduces hospitalization due to influenza in their infants. *Clin Infect Dis* 2010;51:1355-61.
  21. Black SB, Shinefield HR, France EK, et al. Effectiveness of influenza vaccine during pregnancy in preventing hospitalizations and outpatient visits for respiratory illness in pregnant women and their infants. *Am J Perinatol* 2004;21:333-9.
  22. Eick AA, Uyeki TM, Klimov A, et al. Maternal influenza vaccination and effect on influenza virus infection in young infants. *Arch Pediatr Adolesc Med* 2011;165:104-11.
  23. France EK, Smith-Ray R, McClure D, et al. Impact of maternal influenza vaccination during pregnancy on the incidence of acute respiratory illness visits among infants. *Arch Pediatr Adolesc Med* 2006;160:1277-83.
  24. Radin JM, Katz MA, Tempia S, et al. Influenza surveillance in 15 countries in Africa, 2006-2010. *J Infect Dis* 2012;206:Suppl 1:S14-S21.
  25. Poehling KA, Edwards KM, Weinberg GA, et al. The underrecognized burden of influenza in young children. *N Engl J Med* 2006;355:31-40.
  26. Englund JA, Walter E, Black S, et al. Safety and immunogenicity of trivalent inactivated influenza vaccine in infants: a randomized double-blind placebo-controlled study. *Pediatr Infect Dis J* 2010;29:105-10.
  27. Recommended viruses for influenza vaccines for use in the 2011 southern hemisphere influenza season. Geneva: World Health Organization, 2010 ([http://www.who.int/influenza/vaccines/virus/recommendations/201009\\_Recommendation.pdf?ua=1](http://www.who.int/influenza/vaccines/virus/recommendations/201009_Recommendation.pdf?ua=1)).
  28. VAXIGRIP. Inactivated influenza vaccine (Split Virion) product information. France: Sanofi Pasteur, 2011 (<http://www.sanofipasteur.com/en>).
  29. Table for Grading the Severity of Adult and Pediatric Adverse Events Version 1.0 — December 2004 (clarification dated August 2009) (<http://rsc.tech-res.com/safetyandpharmacovigilance>).
  30. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap) — a meta-data-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009;42:377-81.
  31. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* 1972;70:767-77.
  32. Black S, Nicolay U, Vesikari T, et al. Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. *Pediatr Infect Dis J* 2011;30:1081-5.
  33. Häberg SE, Trogstad L, Gunnes N, et al. Risk of fetal death after pandemic influenza virus infection or vaccination. *N Engl J Med* 2013;368:333-40.
  34. Madhi SA, Maskew M, Koen A, et al. Trivalent inactivated influenza vaccine in African adults infected with human immunodeficient virus: double blind, randomized clinical trial of efficacy, immunogenicity, and safety. *Clin Infect Dis* 2011;52:128-37.
  35. Anema A, Mills E, Montaner J, Brownstein JS, Cooper C. Efficacy of influenza vaccination in HIV-positive patients: a systematic review and meta-analysis. *HIV Med* 2008;9:57-61.
  36. Robinson DP, Klein SL. Pregnancy and pregnancy-associated hormones alter immune responses and disease pathogenesis. *Horm Behav* 2012;62:263-71.
  37. Jamieson DJ, Sibailly TS, Sadek R, et al. HIV-1 viral load and other risk factors for mother-to-child transmission of HIV-1 in a breast-feeding population in Cote d'Ivoire. *J Acquir Immune Defic Syndr* 2003;34:430-6.
  38. Tasker SA, O'Brien WA, Treanor JJ, et al. Effects of influenza vaccination in HIV-infected adults: a double-blind, placebo-controlled trial. *Vaccine* 1998;16:1039-42.
  39. Influenza surveillance report — South Africa. National Institute for Communicable Diseases: a division of National Health Laboratory Service, May 2014 ([http://www.nicd.ac.za/?page=seasonal\\_influenza&id=72](http://www.nicd.ac.za/?page=seasonal_influenza&id=72)).

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# Duration of Infant Protection Against Influenza Illness Conferred by Maternal Immunization

## Secondary Analysis of a Randomized Clinical Trial

Marta C. Nunes, PhD; Clare L. Cutland, MD; Stephanie Jones, MD; Andrea Hugo, MD; Richard Madimabe, BTech; Eric A. F. Simões, MD; Adriana Weinberg, MD; Shabir A. Madhi, MD, PhD; for the Maternal Flu Trial Team

**IMPORTANCE** Influenza immunization of women during pregnancy protects the young infants against influenza illness. The duration of this protection remains unclear.

**OBJECTIVE** To evaluate the duration of infant protection conferred by maternal immunization and its association with transplacental antibody transfer.

**DESIGN, SETTING, AND PARTICIPANTS** Infants born to women who participated in a randomized, double-blind, placebo-controlled clinical trial in 2011 and 2012 on the safety, immunogenicity, and efficacy of trivalent inactivated influenza vaccine (IIV3) during pregnancy were followed up during the first 6 months of life for polymerase chain reaction (PCR)-confirmed influenza illness. In a secondary analysis of a subset of infants, hemagglutination inhibition (HAI) antibodies were measured. The study was performed at a single center in South Africa. The secondary analysis was performed in October 2014.

**EXPOSURE** Maternal immunization for influenza.

**MAIN OUTCOMES AND MEASURES** The vaccine's efficacy against PCR-confirmed influenza illness and the percentage of infants with HAI titers of 1:40 or more by age group.

**RESULTS** There were 1026 infants (47.2% female) born to IIV3 recipients and 1023 infants (47.3% female) born to placebo recipients who were included in the analysis of the vaccine's efficacy. The vaccine's efficacy against PCR-confirmed influenza illness was highest among infants 8 weeks of age or younger at 85.6% (95% CI, 38.3%-98.4%) and decreased with increasing age to 25.5% (95% CI, -67.9% to 67.8%) among infants 8 to 16 weeks of age and to 30.3% (95% CI, -154.9% to 82.6%) among infants 16 to 24 weeks of age. Similarly, in the IIV3 group, the percentage of infants with HAI titers of 1:40 or more to the influenza vaccine strains decreased from more than 56% in the first week of life to less than 40% at 16 weeks of age and less than 10.0% at 24 weeks of age.

**CONCLUSIONS AND RELEVANCE** Maternal immunization conferred protection against infection in the infants for a limited period during early life. The lack of protection beyond 8 weeks of age correlated with a decrease in maternally derived antibodies.

**TRIAL REGISTRATION** clinicaltrials.gov Identifier: NCT01306669

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The incidence of influenza illness among infants is high and is associated with a substantial increase in outpatient visits and hospitalizations during the influenza season,<sup>1,2</sup> especially among infants younger than 6 months of age.<sup>2-4</sup> There are also concomitantly high rates of influenza-related deaths in this age group.<sup>5,6</sup> While active annual influenza vaccination is the most efficient mode for the prevention of influenza, current vaccines are poorly immunogenic and not licensed for use for infants younger than 6 months of age. Alternative strategies to prevent influenza illness in young infants include passive protection or cocooning through vaccination of pregnant women and other household members.<sup>7</sup>

We recently reported that immunization with trivalent inactivated influenza vaccine (IIV3) of pregnant women uninfected with the human immunodeficiency virus (HIV) was safe and immunogenic and partially protected the women and their infants with a vaccine efficacy of 50% and 49%, respectively, against polymerase chain reaction (PCR)-confirmed influenza illness during a 24-week follow-up period after delivery.<sup>8</sup> The exact duration of the protection for the infants, however, was never assessed and is generally described during the overall follow-up period (often the first 6 months of life).<sup>8,9</sup>

Furthermore, we also described the kinetics of the transplacentally acquired hemagglutination inhibition (HAI) antibodies in the infants of vaccinated women and determined that the half-life of HAI antibodies in the infants was 43 to 45 days.<sup>10</sup> In this secondary analysis, we report on the secondary and exploratory objectives of the study, which were to determine the percentage of infants born to vaccinated mothers and the percentage of infants born to unvaccinated mothers, both with seroprotective titers from birth to 24 weeks of age, and to evaluate the protection conferred to the infant by maternal immunization stratified by age group.

## Methods

### Study Design

Details of the study have been published elsewhere.<sup>8</sup> In brief, we conducted a randomized, double-blind, placebo-controlled clinical trial of IIV3 for HIV-uninfected pregnant women in Soweto, South Africa.<sup>8</sup> This involved 2 cohorts of HIV-uninfected pregnant women in their second/third trimester who were enrolled prior to the onset of the influenza seasons in 2011 (n = 1060) and 2012 (n = 1056). A subset of these women and their babies were included in the immunogenicity substudy; these participants were recruited from when enrollment started until the desirable sample size was reached. Women and their infants were followed up to 24 weeks post partum. The present report describes results from the infants born to the mothers enrolled in the study. The study used the influenza vaccine recommended by the World Health Organization for the southern hemisphere for both the 2011 and 2012 influenza seasons (A/California/7/2009 [A/H1N1pdm09], A/Victoria/210/2009 [H3N2], and B/Brisbane/60/2008-like virus [B/Victoria lineage];

### Key Points

**Question** For infants, what is the duration of protection against influenza illness conferred by maternal immunization?

**Findings** Infants born to women who participated in a randomized clinical trial of trivalent inactivated influenza vaccine during pregnancy were followed up to determine the vaccine's efficacy against influenza illness and the infants' antibody levels during the first 6 months of life. The vaccine's efficacy was highest among infants 8 weeks of age or younger, and the percentage of infants with seroprotective titers decreased from birth to 6 months.

**Meaning** Maternal immunization conferred protection against infection to the infants for a limited period during early life that correlated with a decrease in maternally derived antibodies.

Vaxigrip; Sanofi Pasteur)<sup>11,12</sup> and sterile 0.9% normal saline solution as placebo.

### Ethical Considerations

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand, registered at ClinicalTrials.gov, and conducted in accordance with Good Clinical Practice guidelines. Mothers provided written informed consent for themselves and their infants.

### Sample Collection and Testing

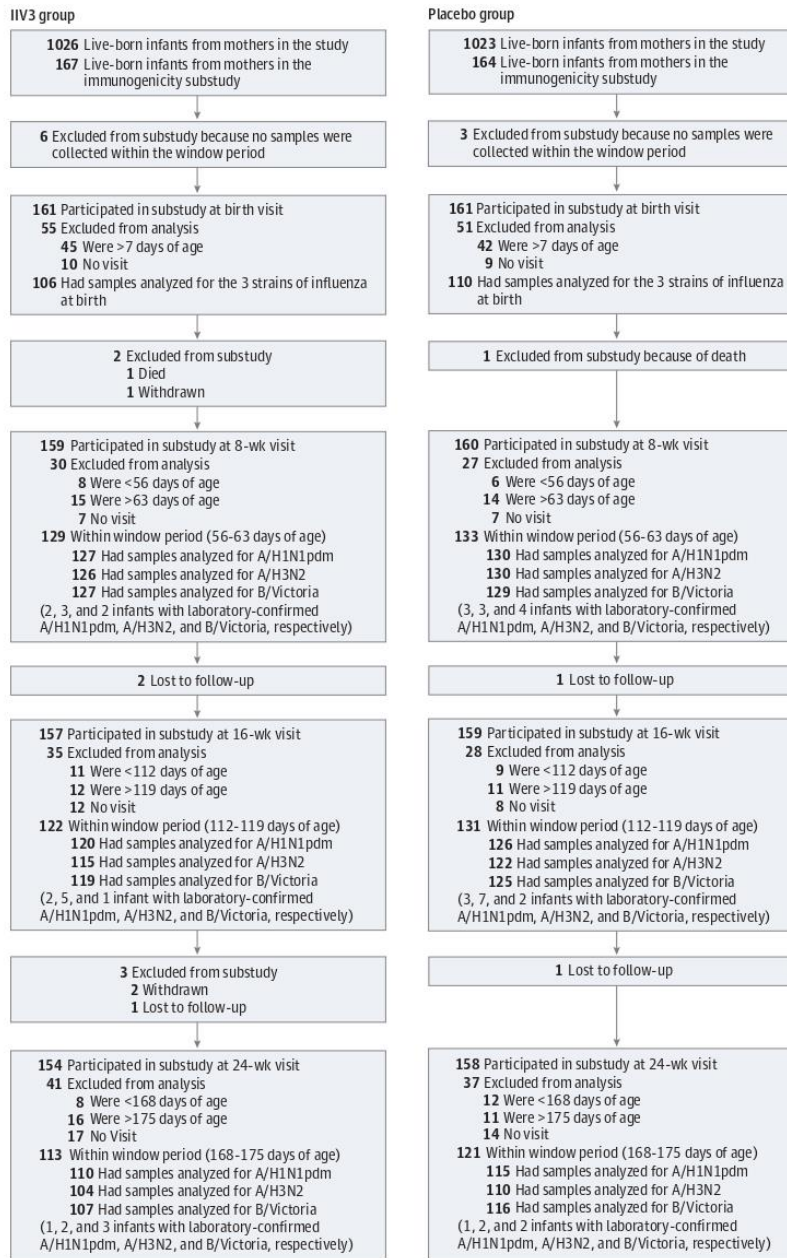
Blood samples were obtained from infants within 7 days of birth and at 8 weeks of age (56-63 days), 16 weeks of age (111-119 days), and 24 weeks of age (168-175 days). The HAI assays were performed as previously described.<sup>13</sup> The percentage of infants with HAI titers of 1:40 or more (putative correlate of protection) was calculated.

Active surveillance for respiratory illness was performed through weekly contact with study participants. Infants with suspected influenza-like illness, as well as those presenting with or hospitalized for any respiratory illness, underwent a procedure to obtain nasopharyngeal aspirate samples. These samples were placed in viral transport medium and tested with a qualitative 2-step real-time reverse transcriptase-PCR assay. Primers and probe sets targeted either the matrix gene or the hemagglutinin gene designed for the universal detection of type A and B influenza viruses, respectively. All influenza A viruses were further subtyped as either H1 or H3, and the influenza B viruses as B/Victoria (homotypic vaccine strain) or B/Yamagata.<sup>14</sup>

### Statistical Analysis

All analyses of study outcomes were performed under the principle of intention to treat, meaning that infants born less than 28 days after their mothers had been vaccinated and preterm infants (ie, <37 weeks of gestational age at birth or a birth weight of <2500 g) were included in the analyses. The immunogenicity analysis included all infants who had blood samples that were obtained within the protocol-defined time periods at each particular time point. Infants who had an episode of PCR-confirmed influenza illness or serological evidence of influenza (ie, ≥4-fold increase in HAI titers between birth and any

Figure 1. Flow Diagram Representing the Number of Infants Analyzed at the Different Immunogenicity Visits



Participants were excluded from the analysis for the relevant strain from the current visit onward. A laboratory-confirmed episode of influenza illness (confirmed by serology or polymerase chain reaction) occurred in the interval between the previous visit and the current visit. IIV3 indicates trivalent inactivated influenza vaccine.

of the subsequent visits) during the follow-up period were excluded from the immunogenicity analyses of the putative strain after the diagnosis of infection. Figure 1 shows the number of infants who contributed data at each study visit. All the infants born to mothers enrolled in the study were included in

the efficacy analyses. The vaccine efficacy was estimated as 1 minus the incidence rate ratio of PCR-confirmed influenza illness in the IIV3 group and placebo group. Incidence rates were calculated as density incidence using Poisson regression and person-time as the denominator. Time-to-event data

Table 1. Infant Characteristics and Time Points of Scheduled Visits

| Variable                                     | Infants, No. (%)       |                        |
|--|------------------------|------------------------|
|  | From IIV3 Recipient    | From Placebo Recipient |
| Full cohort, No.                             | 1026                   | 1023                   |
| Female sex <sup>a</sup>                      | 484 (47.2)             | 484 (47.3)             |
| Born at <37 wk of gestation                  | 108 (10.5)             | 96 (9.4)               |
| Birth weight, mean (SD), <sup>b</sup> kg     | 3.0 (0.5)              | 3.1 (0.5)              |
| Low-birth-weight newborns <sup>b</sup>       | 133 (13.0)             | 122 (12.0)             |
| Time from vaccination to birth, mean (SD), d | 81.5 (35.3)            | 80.6 (35.4)            |
| Follow-up period, median (IQR), d            | 172 (168-175)          | 172 (168-175)          |
| Immunogenicity cohort, No.                   | 161                    | 161                    |
| Female sex                                   | 62 (38.5)              | 75 (46.6)              |
| Born at <37 wk of gestation                  | 17 (10.6)              | 16 (10.0)              |
| Birth weight, median (range), kg             | 3.1 (0.5)              | 3.1 (0.5)              |
| Low-birth-weight newborns                    | 18 (11.2)              | 25 (15.5)              |
| Time from vaccination to birth, mean (SD), d | 76.7 (36.3)            | 81.7 (37.9)            |
| Follow-up period, median (IQR), d            | 169 (168-171)          | 168 (168-170)          |
| Age, <sup>c</sup> median (IQR), d            |                        |                        |
| At first antibody measure                    | 5 (4-6)<br>[106]       | 5 (4-6)<br>[110]       |
| At 8-wk antibody measure                     | 56 (56-57)<br>[129]    | 56 (56-57)<br>[133]    |
| At 16-wk antibody measure                    | 112 (112-114)<br>[122] | 112 (112-114)<br>[131] |
| At 24-wk antibody measure                    | 168 (168-170)<br>[113] | 168 (168-169)<br>[121] |

Abbreviations: IIV3, trivalent inactivated influenza vaccine; IQR, interquartile range.

<sup>a</sup> Missing data for 1 infant in the IIV3 group.

<sup>b</sup> Missing data for 2 infants in the IIV3 group and 2 infants in the placebo group.

<sup>c</sup> Only participants who had their scheduled visits within the study window periods were included in the immunogenicity analyses. The number of infants evaluated at each immunogenicity visit is provided in brackets.

were right censored after the first episode of PCR-confirmed influenza illness or at study termination (maximum 175 days of age). Percentages were compared using the  $\chi^2$  test or the Fisher exact test, and demographic continuous variables were compared using the *t* test or the Mann-Whitney test.  $P < .05$  was considered to be statistically significant. Study data were collected and managed using Research Electronic Data Capture.<sup>15</sup> Analyses were performed using Stata version 12.1 (StataCorp). The South African influenza seasons were defined using the National Institute for Communicable Diseases surveillance data.<sup>16</sup>

## Results

Of 2049 infants included in the vaccine efficacy analysis, 1026 were born to mothers who received IIV3 during pregnancy, and 1023 were born to mothers who received to placebo during pregnancy (Table 1). Infants were born at a mean of 81.0 days (range, 1-175 days) after maternal vaccination and were monitored for influenza illness for a median of 172 days of life (interquartile range, 168-175 days). The baseline demographic characteristics and follow-up times were similar between infants in the IIV3 group and infants in the placebo group. An immunogenicity substudy enrolled 331 infants, including 9 (6 born to IIV3 recipients) who did not have any samples collected within the study's window periods and were excluded from the analysis. The 161 evaluable infants born to IIV3 recipients had similar characteristics and follow-up times as the 161 infants born to placebo recipients (Table 1). Infants

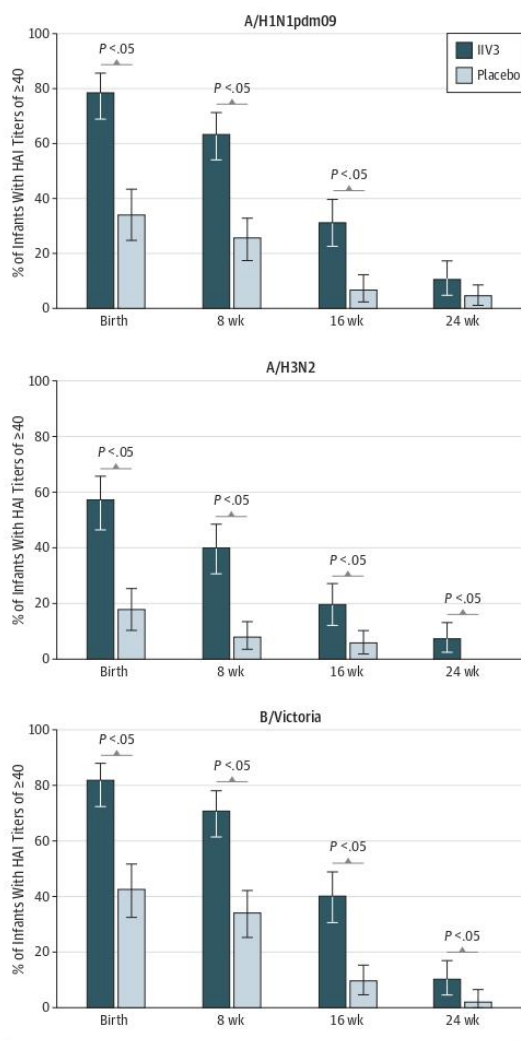
in the immunogenicity substudy had characteristics similar to those of the entire cohort, except for a lower percentage of girls in the substudy (41.7% vs 48.3%;  $P = .03$ ) and a shorter follow-up (168 vs 174 days;  $P < .001$ ) (eTable in the Supplement).

The percentages of infants born to IIV3 recipients with HAI titers of 1:40 or more at birth was 78.3% for the A/H1N1pdm09 strain, 56.6% for the A/H3N2 strain, and 81.1% for the B/Victoria stain; and these percentages were significantly higher than those of infants born to placebo recipients (33.6% for the A/H1N1pdm09 strain, 17.3% for the A/H3N2 strain, and 41.8% for the B/Victoria strain;  $P < .001$  for all comparisons) (Figure 2). The percentage of infants with HAI titers of 1:40 or more decreased in both groups during the first 24 weeks of age. At 16 weeks of age, 39.5%, 19.1%, and 40% of infants born to IIV3 recipients had HAI titers of 1:40 or more for the A/H1N1pdm09 strain, the A/H3N2 strain, and the B/Victoria strain, respectively, and at 24 weeks of age, the percentages were further reduced to 10.0%, 6.7%, and 9.4%, respectively. Corresponding percentages of infants with HAI titers of 1:40 or more in the placebo group were 5% to 9% at 16 weeks ( $P \leq .001$  for all comparisons) and 3.5% ( $P = .06$ ), 0% ( $P = .01$ ), and 1.7% ( $P = .02$ ), respectively, at 24 weeks (Figure 2).

Thirty-seven infants born to placebo recipients and 19 infants born to IIV3 recipients experienced 1 episode of PCR-confirmed influenza illness during the study follow-up period<sup>8</sup> (Table 2). Of the 37 infants born to placebo recipients, 14 were 8 weeks of age or younger, 16 were between 8 and 16 weeks of age, and 7 were between 16 and 24 weeks of age; of



**Figure 2. Percentage of Infants With Hemagglutination Inhibition Titers of 1:40 or More at the Different Study Time Points**



The error bars indicate 95% CIs. HAI, indicates hemagglutination inhibition; IIV3, trivalent inactivated influenza vaccine.

the 19 infants born to IIV3 recipients, 2 were 8 weeks of age or younger, 12 were between 8 and 16 weeks of age, and 5 were between 16 and 24 weeks of age. The vaccine efficacy (against PCR-confirmed influenza illness) was highest when limited to outcomes occurring among infants 8 weeks of age or younger (85.6% [95% CI, 38.3%-98.4%]). The estimated vaccine efficacy gradually decreased with increasing age, including a vaccine efficacy of 53.9% (95% CI, 10.4%-77.4%) for infants 16 weeks of age or younger and 49.5% (95% CI, 9.9%-72.6%) for infants 24 weeks of age or younger. No protective effect of IIV3 was detected if the analysis was restricted to infants between 8 and 16 weeks of age (vaccine

efficacy, 25.5% [95% CI, -67.9% to 67.8%]) or between 16 and 24 weeks of age (vaccine efficacy, 30.3% [95% CI, -154.9% to 82.6%]). Restricting the outcomes to those that occurred during the influenza season yielded similar vaccine efficacy estimates (Table 2).

Seventeen infants in the immunogenicity substudy had an episode of PCR-confirmed influenza illness (7 born to IIV3 recipients and 10 born to placebo recipients). Of these 17 infants, 7 had an episode in which the B/Yamagata strain was detected (2 from the IIV3 group), 5 had an episode in which the A/H3N2 strain was detected (2 from the IIV3 group), 4 had an episode in which the B/Victoria strain was detected (3 from the IIV3 group), and 1 from the placebo group had an episode in which the A/H1N1pdm09 and A/H3N2 strains were simultaneously detected. All 5 infants infected with a homotypic vaccine strain born to IIV3 recipients were between 8 and 16 weeks of age and had a blood sample obtained for determination of HAI titer immediately preceding the influenza episode at an average of 34.6 days (range, 7-56 days). Of the 5 infants in the placebo group infected with a homotypic vaccine strain, 1 was 45 days of age, 2 were between 8 and 16 weeks of age, and 2 were between 16 and 24 weeks of age, and blood samples were available for determination of HAI titer at a mean 27.6 days (range, 15-38 days) before the influenza episode. Table 3 shows that the HAI titers at the immunogenicity visits preceding the episode of PCR-confirmed influenza illness were 1:40 or more in 3 cases (2 in the IIV3 group and 1 in the placebo group) and less than 1:40 in the remaining 8 cases of infection (3 in the IIV3 group and 5 in the placebo group).

## Discussion

We and others have previously demonstrated that the administration of IIV3 during pregnancy confers protection against symptomatic influenza infection to the infants of the vaccinated mothers<sup>8,9</sup>; here we show that the duration of this protection is likely to be limited to the first 8 weeks of age. Several potential mechanisms of protection have been proposed, such as maternal immunization against influenza providing indirect protection of the infant by preventing transmission of influenza virus from the mother to the baby, maternal antibody-mediated protection through transplacental transfer, or maternal antibody-mediated protection through breast milk.<sup>10,17,18</sup> Our study suggests that the most likely mechanism of protection of the infants is through the transplacental transfer of maternal antibodies.<sup>10,19,20</sup>

The concentration of maternally acquired antibodies decreased rapidly in the infants, and by 16 weeks of age, less than 40% of the infants born to IIV3 recipients had HAI titers of 1:40 or more for any of the vaccine strains, mimicking the reduction in vaccine efficacy. Although the HAI titer threshold of 1:40 is generally recognized as corresponding to a 50% reduction in the risk of influenza illness in adults, this may be too low to confer adequate protection in children, and it was estimated that a more appropriate titer for 50% protection in children

**Table 2. Incidence Rates of PCR-Confirmed Influenza Illness and Vaccine Efficacy by Age Group**

| PCR-Confirmed Influenza | IIV3 Group   |                 |                                      | Placebo Group |                 |                                      | Vaccine Efficacy (95% CI) | P Value |
|-------------------------|--------------|-----------------|--------------------------------------|---------------|-----------------|--------------------------------------|---------------------------|---------|
|                         | Infants, No. | Person-Time, mo | Incidence Rate (95% CI) <sup>a</sup> | Infants, No.  | Person-Time, mo | Incidence Rate (95% CI) <sup>a</sup> |                           |         |
| Overall                 |              |                 |                                      |               |                 |                                      |                           |         |
| ≤8 wk                   | 2            | 2083.7          | 1.0 (0.2-3.8)                        | 14            | 2066.4          | 6.8 (4.0-11.4)                       | 85.8 (38.3-98.4)          | .01     |
| >8-16 wk                | 12           | 1818.0          | 6.6 (3.7-11.6)                       | 16            | 1806.5          | 8.9 (5.4-14.5)                       | 25.5 (-67.9 to 67.8)      | .44     |
| >16-24 wk               | 5            | 1670.5          | 3.0 (1.2-7.2)                        | 7             | 1628.8          | 4.3 (2.0-9.0)                        | 30.4 (-154.9 to 82.6)     | .54     |
| ≤16 wk                  | 14           | 3898.0          | 3.6 (2.1-6.1)                        | 30            | 3847.2          | 7.8 (5.5-11.2)                       | 53.9 (10.4-77.4)          | .02     |
| ≤24 wk                  | 19           | 5568.4          | 3.4 (2.2-5.3)                        | 37            | 5476.1          | 6.8 (4.9-9.3)                        | 49.5 (9.9-72.6)           | .02     |
| During influenza season |              |                 |                                      |               |                 |                                      |                           |         |
| ≤8 wk                   | 2            | 1697.8          | 1.2 (0.15-4.3)                       | 14            | 1697.8          | 8.2 (4.5-13.8)                       | 85.4 (36.5-98.4)          | .01     |
| ≤16 wk                  | 14           | 2538.2          | 5.5 (3.0-9.2)                        | 30            | 2534.1          | 11.8 (8.0-16.9)                      | 53.4 (9.4-77.2)           | .02     |
| ≤24 wk                  | 19           | 2766.1          | 6.9 (4.1-10.7)                       | 36            | 2755.1          | 13.1 (9.2-18.0)                      | 47.4 (5.9-71.5)           | .02     |

Abbreviations: IIV3, trivalent inactivated influenza vaccine; PCR, polymerase chain reaction.

<sup>a</sup> Incidence rates calculated as number of cases per 1000 child-months, using person-time between birth and event or end of study.

**Table 3. Data on Antibody Titers in Infants in the Immunogenicity Substudy Who Developed a PCR-Confirmed Influenza Illness**

| Treatment Group, Case No. | Age at Influenza Detection, d | Strain(s) of Influenza Virus | Age Immediately Preceding Collection of Blood Sample, d | HAI Titer  |
|---------------------------|-------------------------------|------------------------------|---|------------|
| IIV3                      |                               |                              |   |            |
| 1                         | 67                            | A/H3N2                       | 59  | 1:160      |
| 2                         | 81                            | A/H3N2                       | 28  | 1:10       |
| 3                         | 105                           | B/Victoria                   | 56  | 1:5        |
| 4                         | 112                           | B/Victoria                   | 56  | 1:10       |
| 5                         | 119                           | B/Victoria                   | 112   | 1:40       |
| Placebo                   |                               |                              |   |            |
| 6                         | 45                            | A/H1N1pdm09; A/H3N2          | 7   | 1:20; 1:80 |
| 7                         | 72                            | A/H3N2                       | 57  | 1:20       |
| 8                         | 82                            | A/H3N2                       | 58  | 1:10       |
| 9                         | 142                           | A/H3N2                       | 118   | 1:5        |
| 10                        | 151                           | B/Victoria                   | 114   | 1:5        |

Abbreviations: HAI, hemagglutination inhibition; IIV3, trivalent inactivated influenza vaccine; PCR, polymerase chain reaction.

older than 6 months of age might be 1:10.<sup>21,22</sup> However, because the threshold of maternally acquired HAI antibodies as a predictor of protection against influenza in young infants is unknown, we used the 1:40 threshold as guidance, anticipating that a higher threshold would predict an even shorter time of protection.

The fact that lower levels of HAI antibodies are associated with reduced protection was also observed in the primary analysis of our IIV3 trials in which both HIV-infected and HIV-uninfected pregnant women participated. In the primary analysis, we showed that HIV-exposed infants had significantly lower HAI titers than HIV-unexposed infants throughout the first 6 months of life and a higher incidence of influenza compared with infants born to uninfected mothers. Although the studies were not powered to show efficacy in the HIV-exposed infants, in the intention-to-treat

analysis, we did not detect any indication that maternal immunization may prevent disease in HIV-exposed infants.<sup>8,10</sup>

The amount of maternal antibodies transferred to the infant is influenced by the interval between immunization and delivery.<sup>23,24</sup> We have previously shown that a higher transplacental antibody transfer was achieved with a longer interval between vaccination and delivery, which suggests that a prolonged cumulative transfer of antibodies leads to higher quantity of antibodies at birth compared with a shorter period during maximal transplacental transfer.<sup>10</sup> Our study finds that, to achieve better infant protection, vaccination during pregnancy should not be delayed until the third trimester.

The main limitation of this study is that the same IIV3 formulation was used in both study years. It would be important to confirm these results in other seasons and in different populations.

## Conclusions

Estimating the period that infants can be protected through maternal vaccination has important implications, especially since currently there is no influenza vaccine licensed for use in infants younger than 6 months of age. To enhance the concentration of the antibodies transferred transplacentally and decrease the period of vulnerability to disease in young

infants, there is the need to find more immunogenic vaccines for pregnant women. Alternatively, more immunogenic vaccines that can generate a protective immune response in infants, beginning at 8 weeks of age, need to be identified. Importantly, whereas protection conferred by vaccination is likely to be mediated by both antibodies and cellular-immune responses in vaccine recipients, passive protection of infants is almost exclusively mediated by antibodies.

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### REFERENCES

- Izurieta HS, Thompson WW, Kramarz P, et al. Influenza and the rates of hospitalization for respiratory disease among infants and young children. *N Engl J Med*. 2000;342(4):232-239.
- Neuzil KM, Mellen BG, Wright PF, Mitchel EF Jr, Griffin MR. The effect of influenza on hospitalizations, outpatient visits, and courses of antibiotics in children. *N Engl J Med*. 2000;342(4):225-231.
- Poehling KA, Edwards KM, Weinberg GA, et al; New Vaccine Surveillance Network. The underrecognized burden of influenza in young children. *N Engl J Med*. 2006;355(1):31-40.
- Poehling KA, Edwards KM, Griffin MR, et al. The burden of influenza in young children, 2004-2009. *Pediatrics*. 2013;131(2):207-216.
- Bhat N, Wright JG, Broder KR, et al; Influenza Special Investigations Team. Influenza-associated deaths among children in the United States, 2003-2004. *N Engl J Med*. 2005;353(24):2559-2567.
- Cohen C, Moyes J, Tempia S, et al. Mortality amongst patients with influenza-associated severe acute respiratory illness, South Africa, 2009-2013. *PLoS One*. 2015;10(3):e0118884.
- Jones C, Heath P. Antenatal immunization. *Hum Vaccin Immunother*. 2014;10(7):2118-2122.
- Madhi SA, Cutland CL, Kuwanda L, et al; Maternal Flu Trial (Matflu) Team. Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med*. 2014;371(10):918-931.
- Zaman K, Roy E, Arifeen SE, et al. Effectiveness of maternal influenza immunization in mothers and infants [published correction appears in *N Engl J Med*. 2009;360(6):648]. *N Engl J Med*. 2008;359(15):1555-1564.
- Nunes MC, Cutland CL, Dighero B, et al; Matflu Team. Kinetics of hemagglutination-inhibiting antibodies following maternal influenza vaccination among mothers with and those without HIV infection and their infants. *J Infect Dis*. 2015;212(12):1976-1987.
- World Health Organization. Recommended viruses for influenza vaccines for use in the 2011 southern hemisphere influenza season. <http://www.who.int/influenza/vaccines/virus/2011south/en/>. Published September 2010. Accessed June 2, 2016.
- Vaxigrip [package insert]. Lyon, France: Sanofi Pasteur; 2011.
- Weinberg A, Song LY, Walker R, et al; IMPAACT P1057 Team. Anti-influenza serum and mucosal antibody responses after administration of live attenuated or inactivated influenza vaccines to HIV-infected children. *J Acquir Immune Defic Syndr*. 2010;55(2):189-196.

14. Influenza Reagent Resource. Atlanta, GA: Centers for Disease Control and Prevention. <https://www.influenzareagentresource.org>. Accessed June 2, 2016.
15. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform.* 2009;42(2):377-381.
16. Respiratory virus surveillance report, South Africa. National Institute for Communicable Diseases: a division of National Health Laboratory Service. [http://www.nicd.ac.za/assets/files/CommDisBul19%2010\(2\)-May%20final2012.pdf](http://www.nicd.ac.za/assets/files/CommDisBul19%2010(2)-May%20final2012.pdf). Accessed June 7, 2016.
17. Maltezos HC, Fotiou A, Antonakopoulos N, et al. Impact of postpartum influenza vaccination of mothers and household contacts in preventing febrile episodes, influenza-like illness, healthcare seeking, and administration of antibiotics in young infants during the 2012-2013 influenza season. *Clin Infect Dis.* 2013;57(11):1520-1526.
18. Schlaudecker EP, Steinhoff MC, Omer SB, et al. IgA and neutralizing antibodies to influenza A virus in human milk: a randomized trial of antenatal influenza immunization. *PLoS One.* 2013;8(8):e70867.
19. Englund JA. The influence of maternal immunization on infant immune responses. *J Comp Pathol.* 2007;137(suppl 1):S16-S19.
20. Steinhoff MC, Omer SB, Roy E, et al. Influenza immunization in pregnancy—antibody responses in mothers and infants. *N Engl J Med.* 2010;362(17):1644-1646.
21. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond).* 1972;70(4):767-777.
22. Black S, Nicolay U, Vesikari T, et al. Hemagglutination inhibition antibody titers as a correlate of protection for inactivated influenza vaccines in children. *Pediatr Infect Dis J.* 2011;30(12):1081-1085.
23. Jackson LA, Patel SM, Swamy GK, et al. Immunogenicity of an inactivated monovalent 2009 H1N1 influenza vaccine in pregnant women. *J Infect Dis.* 2011;204(6):854-863.
24. Blanchard-Rohner G, Meier S, Bel M, et al. Influenza vaccination given at least 2 weeks before delivery to pregnant women facilitates transmission of seroprotective influenza-specific antibodies to the newborn. *Pediatr Infect Dis J.* 2013;32(12):1374-1380.

# Immunogenicity and safety of different dosing schedules of trivalent inactivated influenza vaccine in pregnant women with HIV: a randomised controlled trial



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## Summary

**Background** Standard-dose, seasonal, trivalent, inactivated influenza vaccine induces moderate-to-low haemagglutination-inhibition antibody responses in people living with HIV. This study assessed the immunogenicity and safety of different dosing schedules of inactivated influenza vaccine in pregnant women living with HIV in South Africa.

**Methods** In this double-blind, randomised, controlled trial, we recruited pregnant women with HIV from seven antenatal clinics in Soweto, South Africa. Pregnant women were eligible if they were aged 18–38 years, infected with HIV, and had an estimated gestational age of 12–36 weeks. Women were randomly assigned (1:1:1), using a computer-generated randomisation list, to receive inactivated influenza vaccine containing 15 µg of each of the three seasonal influenza strains for that year, as a single dose, a double dose, or two single doses 1 month apart. Participants and study personnel were masked to group allocation. Haemagglutination-inhibition antibody responses were measured for all groups in the mothers at enrolment and at 1 month after each vaccine dose, and in the single-dose and double-dose groups within 7 days of birth in the neonates. Immunogenicity analyses only included women with visits 28–35 days apart and infants who were born at least 28 days after maternal immunisation. The primary was seroconversion rate to each of the vaccine strains in the mothers 1 month after completion of the dosing schedule, and the primary safety outcomes were frequency of local and systemic reactions. Safety was assessed in mothers and infants until 24 weeks post partum and analysed in all participants who received at least one dose of vaccine. This study is registered with ClinicalTrials.gov, NCT01527825, and is closed to accrual.

**Findings** Between Feb 11, and June 6, 2013, 800 pregnant women living with HIV were enrolled and randomly assigned to the single-dose (n=266), double-dose (n=265), or two-single-doses (n=269) group. In the analysable population, seroconversion rates in mothers 1 month after the final vaccine dose were significantly higher in the double-dose group (n=230; ranging from 29% to 65% for the three vaccine strains) than in the single-dose group (n=230; ranging from 18% to 49%;  $p \leq 0.019$  for the three vaccine strains), but were similar between the two-single-doses group (n=220; ranging from 23% to 52%) and the single-dose group ( $p \geq 0.20$  for the three vaccine strains). Safety outcomes were similar in the three groups, except for more injection-site reactions in recipients in the double-dose group.

**Interpretation** A regimen of double-dose inactivated influenza vaccine gave slightly greater immunogenicity than did a single-dose regimen in pregnant women living with HIV. However, immunogenicity in the double-dose group was still lower than historical data from the same setting in pregnant women without HIV. More immunogenic vaccines are needed for pregnant women living with HIV to enhance transplacental transfer of vaccine-induced protective antibodies to their newborn infants.

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## Introduction

Robust evidence exists to support seasonal influenza vaccination of pregnant women.<sup>1</sup> Randomised controlled trials have shown the efficacy of seasonal trivalent inactivated influenza vaccine during pregnancy in reducing influenza illness in pregnant women and their infants.<sup>2–5</sup> The only randomised controlled trial to date to assess efficacy of influenza vaccination during pregnancy in protecting infants born to women living with HIV was done in South Africa in 2011.<sup>5</sup> This study, although not powered for efficacy, did not find that newborn infants

exposed to HIV had any vaccine-induced protection (vaccine efficacy 26.7%, 95% CI –132.0 to 76.8), possibly due to the modest immune response to inactivated influenza vaccine in pregnant women living with HIV.<sup>5</sup>

In South Africa, after vaccination, pregnant women living with HIV had lower titres of haemagglutination-inhibition antibodies and lower rates of seroconversion (41% vs 92%, to at least one vaccine strain) than did those without HIV.<sup>6</sup> Although transplacental antibody transfer was similar in the women with and without HIV for two of the three vaccine strains contained in the

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**Research in context****Evidence before this study**

Four randomised clinical trials have shown that vaccination of pregnant women against influenza reduces the risk of influenza illness in their infants during the first 6 months of life. However, the only trial that assessed vaccine efficacy among infants born to women living with HIV in South Africa did not detect a protective effect in this group. The lack of vaccine efficacy in infants exposed to HIV might be related to the reduced immune response to vaccination in pregnant women living with HIV. We searched PubMed for publications in English up until May 1, 2019, with no start date restriction using the terms “maternal influenza vaccination HIV”, “maternal influenza immunization HIV”, “pregnancy influenza vaccination HIV”, and “pregnancy influenza immunization HIV”. We found only one study that described immunogenicity in pregnant women with HIV and the antibody levels of their infants after two doses of inactivated A/H1N1/2009 pandemic monovalent vaccine (two doses of 30 µg) during pregnancy. That study reported that a two double doses regimen was moderately immunogenic and that seroprotective titres were present in 67% of mothers and 65% of infants at delivery. After the second dose, only a slight improvement in seroprotection was seen. We did the present analysis to assess if increasing the vaccine antigen

content or administration of a second dose of vaccine would improve the immunogenicity of trivalent inactivated influenza vaccine in pregnant women living with HIV and the protection of their infants against influenza infection compared with a standard single dose of vaccination.

**Added value of this study**

To our knowledge, this is the first randomised clinical trial that has measured the immunogenicity, safety, and efficacy of three different schedules for trivalent inactivated influenza vaccine dosing in pregnant women living with HIV. We found that a double dose of inactivated influenza vaccine had moderately improved immunogenicity in pregnant women living with HIV, although no difference was observed in the incidence of symptomatic influenza in the women or their infants.

**Implications of all the available evidence**

Despite lower antibody responses to influenza vaccination in individuals living with HIV than in previous studies in those without HIV, we found that vaccine efficacy supports the continued vaccination of this vulnerable population. However, continued research is needed to find improved influenza vaccines for pregnant women living with HIV to improve protection of their infants.

vaccine, because of the lower concentration of antibodies after vaccination among the women with HIV, their newborn babies had lower haemagglutination-inhibition antibody titres at birth, and were less likely to have haemagglutination-inhibition titres of 1/40 or higher (putative relative correlate of protection), than the newborn babies of women without HIV (range 43–79% of babies exposed to HIV vs 82–95% of babies not exposed to HIV for the different vaccine strains).<sup>6</sup>

The randomised controlled trials in South Africa also revealed a higher incidence of PCR-confirmed influenza in women with HIV than in women without, both in those given placebo (17.0% vs 3.6%) and those given vaccine (7.0% vs 1.8%).<sup>5</sup> However, overall vaccine efficacy against PCR-confirmed influenza was similar in women living with HIV (57.7%) and those without HIV (50.4%).<sup>5</sup> Several other studies have also reported lower haemagglutination-inhibition antibody responses to inactivated influenza vaccine in individuals living with HIV than in the general population, although with some conflicting results.<sup>7–10</sup> Considering the decreased immunogenicity of standard-dose influenza vaccines in individuals living with HIV, different vaccines or vaccination strategies, such as use of adjuvants, an extra dose of vaccine, or a high-dose inactivated influenza vaccine containing 60 µg of antigen per strain, have been assessed to improve immune response to seasonal vaccines and the 2009 pandemic A/H1N1 monovalent vaccines.<sup>11–17</sup> Adjuvanted vaccines and high-dose inactivated influenza vaccines elicited increased haemagglutination-inhibition titres and improved

seroresponse rates in individuals living with HIV compared with standard doses, but responses were still inferior compared with adults without HIV receiving standard doses of vaccine.<sup>11,12</sup> Furthermore, a second vaccine dose did not consistently increase haemagglutination-inhibition antibody responses.<sup>11,13–15,18</sup>

We hypothesised that increasing the antigen content of a vaccine or administration of a second dose of vaccine would improve the immunogenicity of inactivated influenza vaccine in pregnant women with HIV and the protection of their infants against PCR-confirmed influenza compared with standard-dose inactivated influenza vaccine.

**Methods****Study design and participants**

In this double-blind, randomised, controlled trial, study staff screened pregnant women with documented HIV-1 infection for study participation at seven antenatal clinics in Soweto, South Africa. Soweto is an urban low-income setting with a predominantly black African population of an estimated 1.2 million people located in the outskirts of Johannesburg. HIV prevalence among pregnant women in Soweto has remained stable at 29% since 2009.<sup>19</sup>

Pregnant women were eligible if they were aged 18–38 years, had an estimated gestational age of 12–36 weeks, and were able to understand and comply with the study protocol. Exclusion criteria included any features of WHO clinical category 3 or 4 of AIDS, having been given any other inactivated influenza vaccine during

the current influenza season or received any live licensed vaccine in the past 28 days or an inactivated licensed vaccine in the past 14 days before the study. Full exclusion criteria are in the appendix (p 2). We enrolled participants before the anticipated onset of the influenza season in South Africa. The 2013 South African influenza epidemic period was from April 22 to Oct 13.<sup>20</sup>

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (111114) and done in accordance with Good Clinical Practice guidelines. Mothers provided written informed consent for themselves and their infants. The study protocol is available in the appendix (pp 10–70).

### Randomisation and masking

Participants were randomly assigned (1:1:1) to receive treatment with a single dose of inactivated influenza vaccine followed by placebo (single-dose group), a double dose of inactivated influenza vaccine followed by placebo (double-dose group), or two single doses of inactivated influenza vaccine (two-single-doses group). The second injection for each group was given 1 month after the first injection.

An unmasked study statistician created computer-generated randomisation lists in blocks of 30, with ten blocks in each group (single-dose group, double-dose group, and two-single-doses group), with assignment of consecutive four-digit study numbers to the randomisation list. The randomisation forms were preprinted with the four-digit study numbers and an alphabetical and colour code for vaccine and placebo. Participants were allocated consecutive study numbers in order of enrolment at each study clinic. Participants and study personnel were masked to group allocation. Study medication was prepared by an unmasked pharmacist in syringes filled with either saline or inactivated influenza vaccine as per randomisation and labelled as dominant and non-dominant, and study nurses who enrolled the participants selected syringes matching the codes reflected in the randomisation form (appendix p 3). The pharmacist and study statistician were not involved in participant follow up.

### Procedures

Participants were given a commercially produced influenza vaccine (Vaxigrip; Sanofi-Pasteur, Lyon, France) that contained 15 µg of A/California/7/2009 (A/H1N1pdm09), A/Victoria/361/2011 (A/H3N2), and B/Wisconsin/1/2010 (Yamagata-lineage), as recommended for the southern hemisphere in 2013. Vaxigrip was approved in South Africa for use as a single dose in adults. The study pharmacist prepared inactivated influenza vaccine or sterile 0.9% normal saline solution (placebo) in identical syringes; the two preparations were visually indistinguishable. At enrolment (visit 1), all participants received two injections: inactivated influenza vaccine and placebo were given to the single-dose and

two-single-doses groups, one in each arm, with inactivated influenza vaccine given in the non-dominant arm; and one dose of inactivated influenza vaccine was given in each arm of the participants in the double-dose group (ie, 30 µg of antigen per strain). 1 month later (visit 2), all participants received a single injection on their non-dominant arm, with the single-dose and double-dose groups both given placebo, and the two-single-doses group given another dose of inactivated influenza vaccine.

Participant follow-up was done at the Respiratory and Meningeal Pathogens Research Unit, Soweto, South Africa. We tested immune responses on plasma samples collected from the women immediately before the first injection at visit 1, 1 month after the first injection and immediately before the second injection at visit 2, and 1 month after the second injection at visit 3. We also collected blood within 7 days of birth from the women and their neonates. After review of maternal haemagglutination-inhibition antibody titres, and that no differences were detected between the single-dose and two-single-doses groups, we made a post-hoc decision to analyse only infant blood collected from infants in the single-dose and double-dose groups. Haemagglutination-inhibition antibodies were measured as described previously.<sup>5,21</sup>

We did weekly active surveillance by home visit or telephone call from the time of enrolment through to 24 weeks post partum in mothers and infants for influenza-like illness. The criteria for diagnosing influenza-like illness in the women were onset in the past 7 days of symptoms including: a fever of 38.0°C or higher on oral measurement or history of chills, rigors, or feeling feverish; presence of cough, sore throat, or pharyngitis; presence of myalgia, arthralgia, or headache; or presence of dyspnoea, breathing difficulty, or chest pain when breathing. Criteria used for diagnosing influenza-like illness in infants were any of the following: axillary temperature of 37.8°C or higher or mother's perception that an infant was feverish, or both, without evidence of a non-respiratory localised source, coupled with at least one sign or symptom of acute respiratory illness in the past 72 h; or at least two signs or symptoms of acute respiratory illness in the past 72 h. Signs or symptoms of acute respiratory illness were respiratory rate of 60 or more breaths per min in infants aged 0–2 months and 50 or more breaths per min in infants aged 2–6 months and difficulty breathing reported by the mother, cough, wheezing, runny or congested nose, cyanosis or oxygen saturation below 90% on room air, chest wall in-drawing, grunting on expiration, and pus draining from either ear. Women and infants identified as having influenza-like illness during surveillance visits had respiratory secretions collected within 72 h of illness identification that were tested by influenza PCR. We also investigated by influenza PCR, participants who were admitted to hospital and who had unsolicited study clinic visits due to respiratory illness.

See Online for appendix

For the WHO recommendations see [https://www.who.int/influenza/vaccines/virus/recommendations/2013\\_south/en/](https://www.who.int/influenza/vaccines/virus/recommendations/2013_south/en/)

Details on sample collection, influenza diagnostic and subtyping by PCR have been described previously.<sup>5</sup>

For routine assessment of reactogenicity and safety, the investigator (usually study nurse) observed each participant for 30 min after each injection to monitor immediate adverse events. Additionally, participants were provided with a diary card to document solicited local and systemic reactions for 7 days after each vaccination visit. For injection site reactions, severe reactions were defined as follows: severe tenderness as severe pain in the injected limb that increases when moved or when the movement was reduced, severe redness as redness in an area of 100 mm or larger in size, severe swelling as swelling in an area of 100 mm or larger in size, severe hardness as a large lump being felt wider than half the arm width, severe bruising as a bruise larger than 25 mm in size, and severe itching as itching that requires soothing cream. For systemic reactions, severe reactions were defined as severe weakness or tiredness as being unable to do normal activities during the day, severe headache as a headache requiring medication and being unable to do normal activities during the day; severe fever as an axillary temperature of 39.4°C or higher, and severe joint or muscle pain as severe aching that required medication and that restricted activity. Serious adverse events were collected throughout the study until 24 weeks post partum in women and infants.

### Outcomes

The coprimary study objectives were to assess the relative immunogenicity of a double dose and two single doses of inactivated influenza vaccine compared with a single dose of inactivated influenza vaccine in pregnant women living with HIV to each of the three vaccine strains, and to assess the relative safety of the three dosing schedules. The primary outcome was the seroconversion rate to each of the vaccine strains in mothers 1 month after completion of the dosing schedule.

Secondary objectives reported here were to compare the proportion of neonates born to women in the double-dose group with haemagglutination-inhibition titres of 1/40 or higher at age 7 days or younger (individually for each of the three vaccine strains) with the proportion in the single-dose group, to assess the relative efficacy of the double-dose and two-single-doses regimens compared with the single-dose regimen against PCR-confirmed influenza among the women and their infants until 24 weeks post partum. Additional secondary objectives (appendix p 3) will be reported in the second half of 2020.

Criteria for interpreting haemagglutination-inhibition results included titres of 1/40 or higher as putative measures of relative seroprotection and seroconversion if a four-fold or higher titre increase occurred from baseline to after vaccination with haemagglutination-inhibition antibody titres of 1/40 or higher after vaccination.

Primary safety outcomes were the frequency of solicited local and systemic reactions after a single vaccine dose, a double vaccine dose, or two single doses.

### Statistical analysis

We powered the sample size at 80% with a two-tailed significance level of 0.05 to detect a 30% or higher difference in the proportion of pregnant women living with HIV who seroconverted in the double-dose group or the two-single-doses group, compared with the single-dose group. On the basis of results from our previous study on immune responses to a single dose of inactivated influenza vaccine<sup>5</sup>, we estimated that approximately 44% of women in the single-dose group would have haemagglutination-inhibition antibody titres of 1/40 or higher and a four-fold or higher increase in titres to the least immunogenic strain in the vaccine. The sample size required was 789 women (ie, 263 in each group).

We only included women in the immunogenicity analysis if their vaccination visits were 28–35 days apart and if blood samples were collected 28–35 days after the second injection for those in the two-single-doses group. We only included infants in the immunogenicity analysis if blood samples were collected in the first 7 days of life and if they were born at least 28 days after maternal immunisation. We compared proportions with  $\chi^2$  or Fisher's exact tests and demographic continuous variables with Student's *t* test or the Mann-Whitney test. We estimated geometric mean titres and corresponding 95% CIs by use of logarithmic transformation and compared these between study groups using Student's *t* test. We calculated relative vaccine efficacy against first episode of PCR-confirmed influenza in the intention-to-treat population with the formula  $(1 - I_{sg})/I_{sd}$ , where  $I_{sg}$  is the incidence of cases in the double-dose or two-single-doses group, and  $I_{sd}$  is the incidence of cases in the single-dose group, and we calculated 95% CIs and we tested between-group differences.

The safety analysis set comprised participants who received at least one dose of vaccine. After the first vaccination, we compared the reactions of women who had been given a single dose of vaccine (the single-dose and two-single-doses groups) with those who had been given a double dose (double-dose group), and after the second vaccination we compared women who had received placebo in the single-dose group only with those who had been given another dose of vaccine (the two-single-doses group). We present safety data as incidences with 95% CIs. We did descriptive analyses for the number of maternal, infant, and fetal deaths, and maternal and infant admissions to hospital.

We considered *p* values of less than 0.05 to be significant. We collected and managed study data using Research Electronic Data Capture (REDCap).<sup>22</sup> We did all analyses using STATA version 12.1 (College Station, TX, USA). The study was registered at ClinicalTrials.gov, NCT01527825.



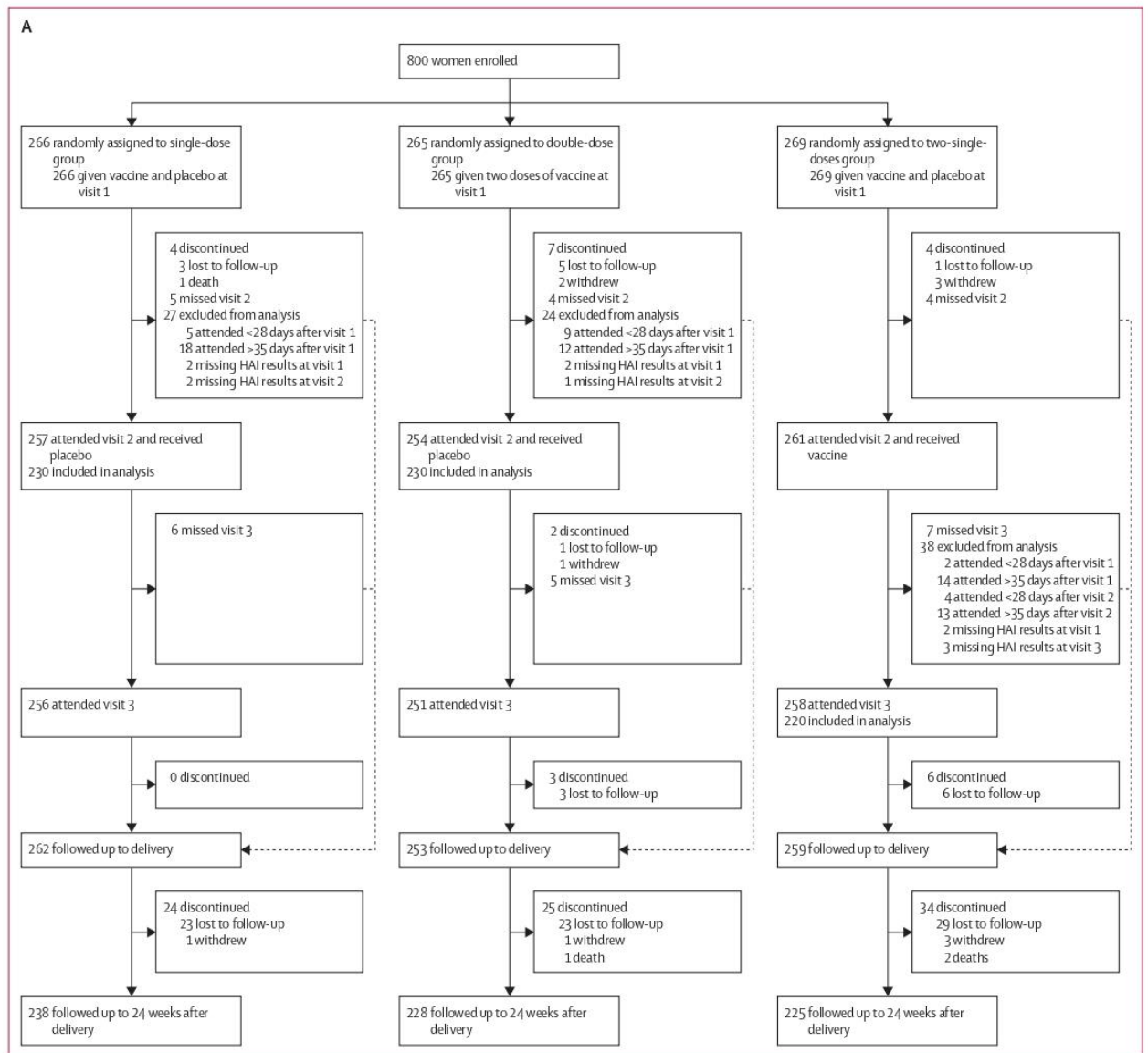
**Role of the funding source**

The funder of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

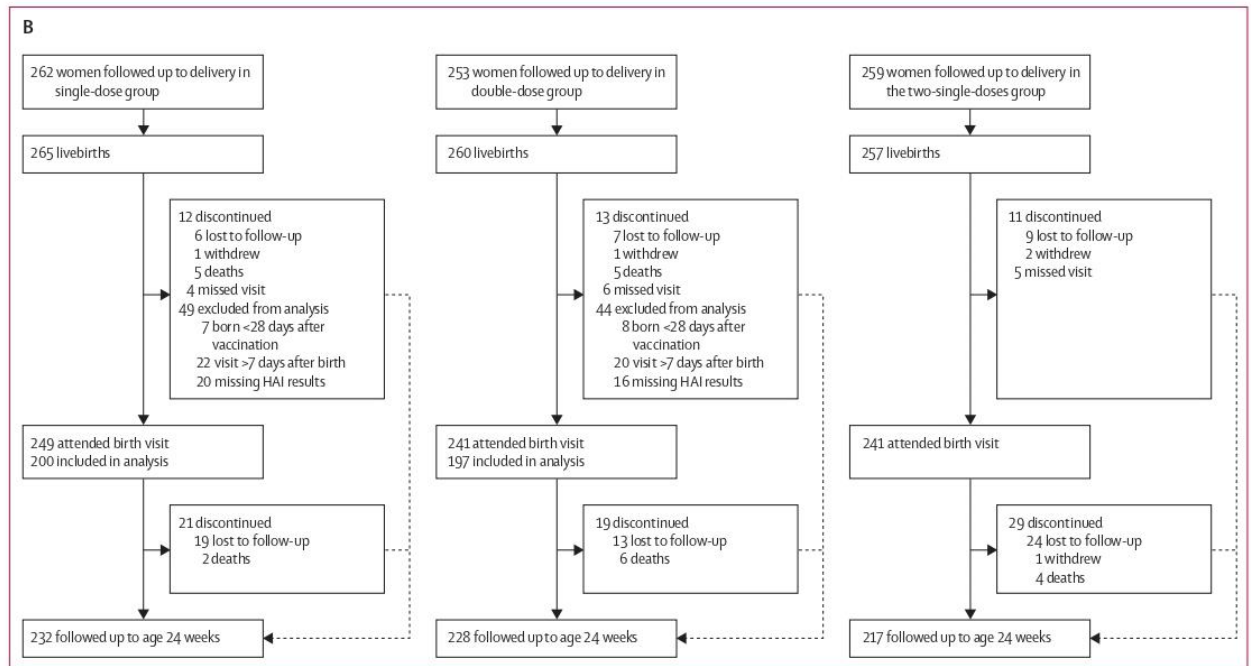
**Results**

Between Feb 11, and June 6, 2013, we enrolled 800 black African pregnant women living with HIV who were

randomly assigned to the single-dose group (n=266), double-dose group (n=265), or two-single-doses group (n=269; figure). Delivery outcomes were established for 774 (97%) of 800 women, including 30 twin pregnancies and one woman in the single-dose group who had a therapeutically indicated medical termination of pregnancy at 25 weeks of gestation (75 days after enrolment). The first infant in the study was born on Feb 22, 2013. 782 livebirths occurred, 136 (17%) of 781 were preterm births (one baby had missing information), 155 (20%) of



(Figure continues on next page)



**Figure: Trial profile for mothers (A) and their infants (B)**

Visit 1 is the enrolment and first injection visit, visit 2 is the second injection visit, and visit 3 is the visit 1 month after the second injection. HAI=haemagglutination-inhibition antibody.

779 neonates had a birthweight of less than 2500 g, and eight (1%) of 681 were infected with HIV. No differences were observed between the three study groups in demographic characteristics, pregnancy outcomes, and birth characteristics of their neonates (table 1). The mean time between the first injection and delivery was 99.6 days (SD 44.8); 145 (19%) women delivered fewer than 28 days after the second injection visit, including 30 who delivered fewer than 28 days after the first injection visit.

Before the first injection, haemagglutination-inhibition antibody geometric mean titres were similar across the three study groups for the three vaccine strains. Also, similar proportions of women in all groups had haemagglutination-inhibition titres of 1/40 or higher for any of the three vaccine strains (tables 2, 3), except for a higher proportion of women in the two-single-doses group for A/H3N2 ( $p=0.023$ ).

1 month after the first injection, haemagglutination-inhibition antibody geometric mean titres increased in women in both the single-dose and double-dose groups but were higher in those in the double-dose group than in those in the single-dose group for all three strains (table 2). Similarly, 1 month after vaccination, seroconversion rates were higher in the double-dose group than in the single-dose group for all three strains (table 2). Furthermore, a higher proportion of women in the double-dose group had haemagglutination-inhibition

titres of 1/40 or higher to each strain than did in the single-dose group ( $p\leq 0.019$  for all comparisons; table 2).

200 infants born to mothers into the single-dose group and 197 infants born to mothers in the double-dose group at least 28 days after vaccination had blood samples collected within the first 7 days of life, with the mean time from birth to blood collection in infants being 3.3 days (SD 1.9) in both groups. We made a post-hoc decision to not analyse blood samples from infants in the two-single-doses group. Infants born to mothers in the double-dose group had higher haemagglutination-inhibition antibody geometric mean titres ( $p=0.046$ ) and a higher proportion had titres of 1/40 or higher for A/H1N1pdm09 than did those born to mothers in the single-dose group ( $p=0.037$ ). We saw no significant differences in haemagglutination-inhibition antibody geometric mean titres or the proportion of infants with titres of 1/40 or higher between the single-dose group and the double-dose group for A/H3N2 and B/Yamagata (table 2). Similar results were obtained when the 41 eligible preterm infants were excluded in a post-hoc analysis, except that the significant differences for A/H1N1pdm09 were no longer detected (data not shown). Additionally, we saw no difference in geometric mean titres and the proportion of infants with haemagglutination-inhibition antibody titres of 1/40 or higher between preterm and term infants overall and by study group (data not shown).

|  | Single-dose group<br>(n=266)  | Double-dose group<br>(n=265) | Two-single-doses group<br>(n=269) | Total population<br>(n=800)   |
|--|-------------------------------|------------------------------|-----------------------------------|-------------------------------|
| <b>Demographic and clinical characteristics</b>        |                               |                              |                                   |                               |
| Age, years   | 28.8 (5.0)                    | 29.0 (4.8)                   | 28.4 (4.9)                        | 28.7 (4.9)                    |
| Gestational age at enrolment, weeks                    | 23.4 (6.0)                    | 24.2 (6.1)                   | 23.8 (5.6)                        | 23.8 (5.9)                    |
| Primigravida women                                     | 30 (11%)                      | 40 (15%)                     | 31 (12%)                          | 101 (13%)                     |
| CD4 count within 2 weeks of enrolment                  |                               |                              |                                   |                               |
| n  | 257                           | 255                          | 263                               | 775                           |
| Mean, cells per $\mu$ L                                | 458.7 (260.5)                 | 463.9 (256.5)                | 454.4 (227.6)                     | 459.0 (248.2)                 |
| <350 cells per $\mu$ L                                 | 99/257 (39%)                  | 91/255 (36%)                 | 98/263 (37%)                      | 288/775 (37%)                 |
| HIV viral load within 2 weeks of enrolment             |                               |                              |                                   |                               |
| n  | 245                           | 243                          | 249                               | 737                           |
| Median, copies per mL*                                 | 4918 (475–34 863)             | 7298 (511–32 360)            | 5152 (711–28 486)                 | 6066 (524–33 044)             |
| <40 copies per mL                                      | 65/245 (27%)                  | 59/243 (24%)                 | 72/249 (29%)                      | 196/737 (27%)                 |
| Antiretroviral therapy at enrolment                    | 248/263 (94%)                 | 250/262 (95%)                | 256/267 (96%)                     | 754/792 (95%)                 |
| On antiretroviral therapy for $\geq$ 30 days           | 129/255 (51%)                 | 126/255 (49%)                | 143/265 (54%)                     | 398/775 (51%)                 |
| <b>Delivery outcomes</b>                               |                               |                              |                                   |                               |
| Known delivery outcome                                 | 262 (98%) <sup>†</sup>        | 253 (95%)                    | 259 (96%)                         | 774 (97%) <sup>†</sup>        |
| Number of twin pregnancies                             | 9/262 (3%)                    | 12/253 (5%)                  | 9/259 (3%)                        | 30/774 (4%)                   |
| Days between first injection and delivery <sup>‡</sup> | 102.9 (45.9)                  | 96.9 (45.6)                  | 98.8 (42.8)                       | 99.6 (44.8)                   |
| Delivered <28 days after first injection <sup>‡</sup>  | 9/262 (3%)                    | 8/253 (3%)                   | 13/259 (5%)                       | 30/774 (4%)                   |
| Delivered <28 days after second injection <sup>‡</sup> | 47/262 (18%)                  | 54/253 (21%)                 | 44/259 (17%)                      | 145/774 (19%)                 |
| Birth outcomes known                                   | 270 <sup>‡</sup> <sub>5</sub> | 265                          | 268 <sup>¶</sup> <sub>1</sub>     | 803 <sup>‡</sup> <sub>5</sub> |
| Fetal deaths   | 5/270 (2%)                    | 5/265 (2%)                   | 11/268 (4%)                       | 21/803 (3%)                   |
| Livebirths   | 265                           | 260                          | 257                               | 782                           |
| Births <37 weeks of gestation <sup>  </sup>            | 48/264 (18%)                  | 38/260 (15%)                 | 50/257 (19%)                      | 136/781 (17%)                 |
| Birthweight, kg <sup>  </sup>                          | 2.9 (0.6)                     | 2.9 (0.6)                    | 2.9 (0.6)                         | 2.9 (0.6)                     |
| Neonates with <2500 g birthweight <sup>  </sup>        | 52/263 (20%)                  | 53/260 (20%)                 | 50/256 (20%)                      | 155/779 (20%)                 |
| Infants exclusively breastfed at birth visit           | 103/247 (42%)                 | 103/235 (44%)                | 96/239 (40%)                      | 302/721 (42%)                 |

Data are mean (SD), median (IQR), n (%), or n/N (%). \*Excludes participants with a viral load of <40 copies per mL HIV. <sup>†</sup>Excluding one woman who died while pregnant. <sup>‡</sup>Based on women with known delivery outcomes. <sup>§</sup>Excluding one woman who had a therapeutically indicated medical termination of pregnancy. <sup>¶</sup>14 birth outcomes occurred before second vaccination visit. <sup>||</sup>Based on livebirths.

**Table 1: Maternal demographic and clinical characteristics at time of enrolment and delivery outcomes**

In mothers, haemagglutination-inhibition geometric mean titres were similar 1 month after the completion of the vaccination series between the single-dose and two-single-doses groups, except for higher geometric mean titres for B/Yamagata after the second dose in the two-single-doses group (table 3). 1 month after completion of full vaccination series, we saw no differences in the proportion of women with titres of 1/40 or higher or who had seroconverted between the single-dose and two-single-doses groups for any of the three vaccine strains (table 3). Notably, we saw no increase in haemagglutination-inhibition antibody geometric mean titres or the proportion of women with titres of 1/40 or higher 1 month after the second dose of vaccine in the two-single-doses group compared with 1 month after the first dose in this group (appendix p 5).

65 (8%) of 800 women had at least one episode of PCR-confirmed influenza, with two women, both in the single-dose group, having two distinct episodes by A/H1N1pdm09 and B/Yamagata more than 2 months

apart. 42 PCR-confirmed influenza episodes were detected during pregnancy, with one woman having two episodes while pregnant. PCR-confirmed A/H1N1pdm09 was detected in 34 women, PCR-confirmed A/H3N2 in 22 women, and PCR-confirmed B/Yamagata in 12 women, including one woman in whom both A/H3N2 and B/Yamagata were co-detected. Three women, one in each study group, had PCR-confirmed episodes that were detected fewer than 14 days after the first vaccination visit; and an additional four women in the two-single-doses group had a PCR-confirmed influenza episode either before (n=2) or fewer than 14 days after (n=2) the second dose of vaccine. We saw no differences in the incidence of first episode of PCR-confirmed influenza between women in the single-dose group compared with the double-dose group (p=0.88) or the two-single-doses group (p=0.67) overall or by individual influenza strain (table 4).

23 PCR-confirmed episodes of influenza (seven of A/H1N1pdm09, five of A/H3N2, ten of B/Yamagata, and one B/Victoria) were recorded among 21 infants. Two infants in the two-single-doses group had

|   | A/H1N1pdm09       |                   |         | A/H3N2            |                   |         | B/Yamagata        |                   |         |
|---|-------------------|-------------------|---------|-------------------|-------------------|---------|-------------------|-------------------|---------|
|   | Single-dose group | Double-dose group | p value | Single-dose group | Double-dose group | p value | Single-dose group | Double-dose group | p value |
| Mothers*                                    | 230               | 230               | ..      | 230               | 230               | ..      | 230               | 230               | ..      |
| HAI GMTs at baseline                        | 9.6 (8.5–10.9)    | 9.5 (8.4–10.8)    | 0.92    | 11.8 (10.5–13.3)  | 13.3 (11.7–15.1)  | 0.19    | 5.9 (5.6–6.2)     | 6.1 (5.7–6.5)     | 0.39    |
| HAI GMTs at 28–35 days after visit 1†       | 39.5 (33.1–47.1)  | 60.8 (51.2–72.2)  | 0.001   | 37.8 (32.3–44.1)  | 52.7 (45.4–61.1)  | 0.003   | 13.0 (11.6–14.5)  | 16.6 (14.7–18.8)  | 0.004   |
| HAI $\geq$ 1/40 at baseline                 | 33 (14%)          | 29 (13%)          | 0.59    | 34 (15%)          | 50 (22%)          | 0.053   | 4 (2%)            | 4 (2%)            | 1.0     |
| HAI $\geq$ 1/40 at 28–35 days after visit 1 | 142 (62%)         | 171 (74%)         | 0.004   | 131 (57%)         | 163 (71%)         | 0.002   | 46 (20%)          | 71 (31%)          | 0.007   |
| Seroconversion at 28–35 days after visit 1  | 113 (49%)         | 150 (65%)         | <0.001  | 95 (41%)          | 120 (52%)         | 0.019   | 41 (18%)          | 67 (29%)          | 0.004   |
| Infants overall‡                            | 200               | 197               | ..      | 200               | 197               | ..      | 200               | 197               | ..      |
| HAI GMTs at age $\leq$ 7 days               | 29.7 (24.7–35.5)  | 38.5 (32.1–46.1)  | 0.046   | 23.3 (19.7–27.5)  | 26.2 (22.3–30.9)  | 0.32    | 13.0 (11.7–14.4)  | 14.4 (12.8–16.2)  | 0.20    |
| HAI $\geq$ 1/40 at age $\leq$ 7 days        | 99 (50%)          | 118 (60%)         | 0.037   | 77 (39%)          | 90 (46%)          | 0.15    | 32 (16%)          | 37 (19%)          | 0.47    |

Data are n, n (%), or GMTs with 95% CI in parentheses. GMTs=geometric means titres. HAI=haemagglutination inhibition antibody. \*Only women who attended the visits within the protocol-defined time periods were included. †GMTs significantly higher than at baseline for all comparisons. ‡Only infants born  $\geq$ 28 days after their mothers had been vaccinated and with blood sample collected in the first 7 days of life were included.

Table 2: Immune responses in pregnant women living with HIV and antibody levels in their infants, in the single-dose and double-dose groups

|   | A/H1N1pdm09               |                                |         | A/H3N2                    |                                |         | B/Yamagata                |                                |         |
|---|---------------------------|--------------------------------|---------|---------------------------|--------------------------------|---------|---------------------------|--------------------------------|---------|
|   | Single-dose group (n=230) | Two-single-doses group (n=220) | p value | Single-dose group (n=230) | Two-single-doses group (n=220) | p value | Single-dose group (n=230) | Two-single-doses group (n=220) | p value |
| HAI GMTs at baseline  | 9.6 (8.5–10.9)            | 9.5 (8.4–10.8)                 | 0.76    | 11.8 (10.5–13.3)          | 12.5 (11.0–14.2)               | 0.99    | 5.9 (5.6–6.2)             | 5.9 (5.6–6.2)                  | 0.94    |
| HAI GMTs at 28–35 days after completion of full vaccine series*†                | 39.5 (33.1–47.1)          | 46.7 (39.7–54.9)               | 0.17    | 37.8 (32.3–44.1)          | 44.1 (37.5–51.9)               | 0.18    | 13.0 (11.6–14.5)          | 15.5 (13.9–17.3)               | 0.029   |
| HAI antibody $\geq$ 1/40 at baseline  | 33 (14%)                  | 31 (14%)                       | 0.94    | 34 (15%)                  | 51 (23%)                       | 0.023   | 4 (2%)                    | 2 (1%)                         | 0.59    |
| HAI antibody $\geq$ 1/40 at 28–35 days after completion of full vaccine series† | 142 (62%)                 | 148 (67%)                      | 0.22    | 131 (57%)                 | 145 (66%)                      | 0.051   | 46 (20%)                  | 54 (25%)                       | 0.25    |
| Seroconversion at 28–35 days after first vaccination†                           | 113 (49%)                 | 115 (52%)                      | 0.51    | 95 (41%)                  | 103 (47%)                      | 0.24    | 41 (18%)                  | 50 (23%)                       | 0.20    |

Data are n (%) and GMTs with 95% CI in parentheses. Only women who attended the visits within the protocol-defined time periods are included. GMTs=geometric means titres. HAI=haemagglutination inhibition antibody. \*GMTs significantly higher than at baseline for all comparisons. †For single-dose group assessed at 28–35 days after first injection; for two-single-doses group assessed at 28–35 days after second injection.

Table 3: Immune responses of pregnant women living with HIV in the single-dose and two-single-doses groups

two distinct episodes 2 or more months apart (one infant was infected by A/H3N2 and B/Yamagata and the other had two episodes of B/Yamagata infection at age 45 days and 94 days). We saw no differences in the incidence of PCR-confirmed influenza among infants in the single-dose group compared with the double-dose group ( $p=0.99$ ) or the two-single-doses group ( $p=0.28$ ; table 4).

The incidence of one or more local solicited reaction was greater in the double-dose group (47.8% in the non-dominant arm and 38.8% in the dominant arm) than in the single-dose and two-single-doses groups (38.1% non-dominant arm and 26.7% dominant arm;  $p\leq 0.010$  for both), but we saw no difference in the proportion who had severe local reactions (table 5). However, women who were given a single dose of vaccine had a higher frequency of at least one severe solicited systemic reaction than did those given a double dose ( $p=0.022$ ), mainly headache and weakness or tiredness (table 5). After the second injection, we saw no differences in solicited local and

systemic reactions between women in the single-dose group and the two-single-doses group (table 5).

Four maternal deaths occurred during the study period (ie, until 24 weeks after delivery), one in the single-dose group, one in the double-dose group, and two in the two-single-doses group; none of these deaths were attributed to the vaccine (figure; appendix p 6). 22 infant deaths occurred up to age 24 weeks, of which seven were in the single-dose group, 11 were in the double-dose group, and four were in the two-single-doses group (figure; appendix pp 8–9). None of the infant deaths were attributed to maternal vaccination. Furthermore, 21 fetal deaths occurred, 11 in women in the two-single-doses group (of which three occurred before the second vaccination visit), five in the single-dose group, and five in the double-dose group (appendix p 7). Four fetal deaths occurred within 14 days of maternal vaccination, one in the single-dose group and three in the two-single-doses group (two after the first dose and one after the second

dose), and were consequently deemed possibly related to maternal vaccination. However, this association was only based on temporal associations to vaccination (adverse event within 14 days of vaccination), which was decided a priori. The three women (including one twin pregnancy) did not report any local or systemic reactions after vaccination that could indicate a causative effect of the vaccine (appendix p 7). We saw no differences in the proportion of maternal, infant, or fetal deaths, or infant birthweights between the single-dose group and the other two study groups (table 1).

## Discussion

In pregnant women living with HIV, the use of a double dose of inactivated influenza vaccine slightly improved immunogenicity compared with a single dose, but at 1 month after vaccination fewer than 66% of women in the double-dose group seroconverted to the most immunogenic vaccine strain (ie, A/H1N1pdm09), which was lower than the 92% seroconversion seen in our 2011 cohort of pregnant women without HIV who were given a standard single dose of inactivated influenza vaccine.<sup>5</sup> A second standard inactivated influenza vaccine dose did not

|                           | Single-dose group | Double-dose group | Relative vaccine efficacy | p value | Two-single-doses group | Relative vaccine efficacy | p value |
|---------------------------|-------------------|-------------------|---------------------------|---------|------------------------|---------------------------|---------|
| Mothers                   | 266               | 265               | ..                        | ..      | 269                    | ..                        | ..      |
| PCR-confirmed influenza   | 21 (8%)           | 20 (8%)           | 4.4% (-72.2 to 46.9)      | 0.88    | 24 (9%)                | -13.0% (-98.0 to 35.5)    | 0.67    |
| PCR-confirmed A/H1N1pdm09 | 14 (5%)           | 8 (3%)            | 42.6% (-34.4 to 75.6)     | 0.19    | 12 (4%)                | 13.6% (-83.2 to 59.3)     | 0.70    |
| PCR-confirmed A/H3N2      | 4 (2%)            | 8 (3%)            | -100.8% (-558.6 to 38.8)  | 0.26    | 10 (4%)                | -146.3% (-675.4 to 21.8)  | 0.17    |
| PCR-confirmed B/Yamagata  | 5 (2%)            | 5 (2%)            | -0.4% (-242.7 to 70.6)    | 0.99    | 2 (1%)                 | 60.4% (-102.1 to 92.3)    | 0.25    |
| Infants                   | 265               | 260               | ..                        | ..      | 257                    | ..                        | ..      |
| PCR-confirmed influenza   | 6 (2%)            | 5 (2%)            | 15.1% (-174.9 to 73.8)    | 0.99    | 10 (4%)                | -71.9% (-366.0 to 36.6)   | 0.28    |

Data are n, n (%), or relative vaccine efficacy compared with single-dose group, with 95% CI in parentheses. Among mothers, the overall number of cases of PCR-confirmed influenza are lower than the sum of the individual strains because one woman in the double-dose group had a double infection with B/Yamagata and A/H3N2 and two women in the single-dose group had two different PCR-confirmed influenza infections, both women with A/H1N1pdm09 and B/Yamagata.

**Table 4: Relative efficacy of a double dose or two single doses compared with a single dose of vaccine against first episode of PCR-confirmed influenza in HIV-infected pregnant women and their infants until 24 weeks after birth**

|  | First week after first vaccination              |                           |         | First week after second vaccination |                                |         |
|--|---|---------------------------|---------|-------------------------------------|--------------------------------|---------|
|  | Single-dose and two-single-doses groups (n=517) | Double-dose group (n=255) | p value | Single-dose group (placebo; n=240)  | Two-single-doses group (n=238) | p value |
| <b>Non-dominant arm local injection-site reactions</b> |   |                           |         |                                     |                                |         |
| <b>Tenderness</b>                                      |   |                           |         |                                     |                                |         |
| Any  | 24.6% (20.9-28.5)                               | 28.3% (22.8-34.2)         | 0.27    | 15.0% (10.7-20.2)                   | 18.5% (13.8-24.0)              | 0.31    |
| Severe   | 2.7% (1.5-4.5)                                  | 1.6% (0.4-4.0)            | 0.45    | 2.1% (0.7-4.8)                      | 0.4% (<0.1-2.3)                | 0.22    |
| <b>Redness</b>   |   |                           |         |                                     |                                |         |
| Any  | 7.0% (4.9-9.5)                                  | 7.8% (4.9-11.9)           | 0.66    | 2.1% (0.7-4.8)                      | 3.4% (1.5-6.5)                 | 0.42    |
| Severe   | 1.9% (0.9-3.5)                                  | 2.0% (0.6-4.5)            | 0.99    | 1.3% (0.3-3.6)                      | 0.4% (<0.1-2.3)                | 0.62    |
| <b>Swelling</b>  |   |                           |         |                                     |                                |         |
| Any  | 8.3% (6.1-11.0)                                 | 6.3% (3.6-10.0)           | 0.32    | 2.9% (1.2-5.9)                      | 4.2% (2.0-7.6)                 | 0.45    |
| Severe   | 1.7% (0.8-3.3)                                  | 2.0% (0.6-4.5)            | 0.78    | 1.7% (0.5-4.2)                      | 0.4% (<0.1-2.3)                | 0.37    |
| <b>Hardness</b>  |   |                           |         |                                     |                                |         |
| Any  | 19.9% (16.6-23.6)                               | 22.0% (17.0-27.5)         | 0.51    | 11.7% (7.9-16.4)                    | 15.6% (11.2-20.8)              | 0.22    |
| Severe   | 2.1% (1.1-3.8)                                  | 1.2% (0.2-3.4)            | 0.57    | 2.1% (0.7-4.8)                      | 0.4% (<0.1-2.3)                | 0.22    |
| <b>Bruising</b>  |   |                           |         |                                     |                                |         |
| Any  | 12.4% (10.0-15.5)                               | 9.8% (6.4-14.1)           | 0.29    | 8.8% (5.5-13.1)                     | 9.2% (5.9-13.7)                | 0.85    |
| Severe   | 0.8% (0.2-2.0)                                  | 0.8% (<0.1-2.8)           | 1.0     | 0.4% (<0.1-2.3)                     | 0                              | 1.0     |
| <b>Itching</b>   |   |                           |         |                                     |                                |         |
| Any  | 19.0% (15.7-22.6)                               | 22.8% (17.7-28.4)         | 0.22    | 12.5% (8.6-17.4)                    | 13.0% (9.0-18.0)               | 0.86    |
| Severe   | 1.9% (0.9-3.5)                                  | 0.4% (<0.1-2.2)           | 0.11    | 0                                   | 0.8% (0.1-3.0)                 | 0.50    |
| <b>At least one local reaction</b>                     |   |                           |         |                                     |                                |         |
| Any  | 38.1% (33.9-42.4)                               | 47.8% (41.6-54.1)         | 0.010   | 23.8% (18.5-29.6)                   | 28.2% (22.5-34.3)              | 0.27    |
| Severe   | 6.6% (4.6-9.1)                                  | 4.7% (2.5-8.1)            | 0.30    | 4.2% (2.0-7.5)                      | 1.7% (0.5-4.2)                 | 0.17    |

(Table 5 continues on next page)

|  | First week after first vaccination              |                           |         | First week after second vaccination |                                |         |
|--|---|---------------------------|---------|-------------------------------------|--------------------------------|---------|
|  | Single-dose and two-single-doses groups (n=517) | Double-dose group (n=255) | p value | Single-dose group (placebo; n=240)  | Two-single-doses group (n=238) | p value |
| (Continued from previous page)   |   |                           |         |                                     |                                |         |
| <b>Dominant arm local injection-site reactions</b>   |   |                           |         |                                     |                                |         |
| Tenderness   |   |                           |         |                                     |                                |         |
| Any  | 19.5% (16.2-23.2)                               | 22.0% (17.0-27.5)         | 0.43    | ..                                  | ..                             | ..      |
| Severe   | 1.4% (0.1-2.5)                                  | 1.2% (0.2-3.4)            | 0.99    | ..                                  | ..                             | ..      |
| Redness  |   |                           |         |                                     |                                |         |
| Any  | 4.5% (2.8-6.6)                                  | 6.3% (3.6-10.0)           | 0.28    | ..                                  | ..                             | ..      |
| Severe   | 0.6% (0.1-1.7)                                  | 2.4% (0.9-5.0)            | 0.066   | ..                                  | ..                             | ..      |
| Swelling   |   |                           |         |                                     |                                |         |
| Any  | 4.1% (2.5-6.1)                                  | 6.3% (3.6-10.0)           | 0.18    | ..                                  | ..                             | ..      |
| Severe   | 0.8% (0.2-2.0)                                  | 2.0% (0.6-4.5)            | 0.17    | ..                                  | ..                             | ..      |
| Hardness   |   |                           |         |                                     |                                |         |
| Any  | 14.1% (11.2-17.4)                               | 18.0% (13.5-23.3)         | 0.16    | ..                                  | ..                             | ..      |
| Severe   | 0.8% (0.2-2.0)                                  | 1.2% (0.2-3.4)            | 0.69    | ..                                  | ..                             | ..      |
| Bruising   |   |                           |         |                                     |                                |         |
| Any  | 9.3% (6.9-12.1)                                 | 9.4% (6.1-13.7)           | 0.95    | ..                                  | ..                             | ..      |
| Severe   | 0.4% (0.1-1.4)                                  | 0                         | 1.0     | ..                                  | ..                             | ..      |
| Itching  |   |                           |         |                                     |                                |         |
| Any  | 12.8% (10.0-16.0)                               | 18.0% (13.5-23.3)         | 0.050   | ..                                  | ..                             | ..      |
| Severe   | 1.2% (0.4-2.5)                                  | 1.2% (0.2-3.4)            | 1.0     | ..                                  | ..                             | ..      |
| At least one local reaction  |   |                           |         |                                     |                                |         |
| Any  | 26.7% (22.9-30.7)                               | 38.8% (32.8-45.1)         | 0.001   | ..                                  | ..                             | ..      |
| Severe   | 3.7% (2.2-5.7)                                  | 5.1% (2.7-8.6)            | 0.35    | ..                                  | ..                             | ..      |
| <b>Systemic reactions</b>  |   |                           |         |                                     |                                |         |
| Weakness or tiredness  |   |                           |         |                                     |                                |         |
| Any  | 35.8% (31.6-40.1)                               | 34.9% (29.1-41.1)         | 0.81    | 27.5% (22.0-33.6)                   | 24.0% (18.7-29.9)              | 0.38    |
| Severe   | 6.0% (4.1-8.4)                                  | 2.8% (1.1-5.6)            | 0.050   | 6.3% (3.5-10.1)                     | 2.9% (1.2-6.0)                 | 0.084   |
| Headache   |   |                           |         |                                     |                                |         |
| Any  | 35.2% (31.1-39.5)                               | 32.6% (26.8-38.7)         | 0.47    | 30.0% (24.3-36.2)                   | 24.4% (19.1-30.3)              | 0.17    |
| Severe   | 6.0% (4.1-8.4)                                  | 2.0% (0.6-4.5)            | 0.011   | 4.2% (2.0-7.5)                      | 2.5% (0.9-5.4)                 | 0.32    |
| Fever  |   |                           |         |                                     |                                |         |
| Any  | 4.3% (2.7-6.4)                                  | 1.6% (0.4-4.0)            | 0.057   | 3.8% (1.7-7.0)                      | 3.8% (1.7-7.1)                 | 0.99    |
| Severe   | 0.8% (0.2-2.0)                                  | 0                         | 0.31    | 0.4% (<0.1-2.3)                     | 0.8% (0.1-3.0)                 | 0.62    |
| Joint pain   |   |                           |         |                                     |                                |         |
| Any  | 19.5% (16.2-23.2)                               | 20.0% (15.3-25.4)         | 0.88    | 16.3% (11.8-21.5)                   | 11.8% (8.0-16.5)               | 0.16    |
| Severe   | 2.7% (1.5-4.5)                                  | 1.2% (0.2-3.4)            | 0.20    | 2.1% (0.7-4.8)                      | 1.3% (0.3-3.6)                 | 0.72    |
| Muscle pain  |   |                           |         |                                     |                                |         |
| Any  | 17.8% (14.6-21.4)                               | 21.2% (16.3-26.7)         | 0.26    | 18.3% (13.6-23.8)                   | 11.3% (7.6-16.1)               | 0.032   |
| Severe   | 2.3% (1.2-4.0)                                  | 2.0% (0.6-4.5)            | 1.0     | 2.1% (0.7-4.8)                      | 0.8% (0.1-3.0)                 | 0.45    |
| Rigors   |   |                           |         |                                     |                                |         |
| Any  | 5.8% (3.9-8.2)                                  | 5.5% (3.0-9.0)            | 0.86    | 3.8% (1.7-7.0)                      | 3.8% (1.7-7.1)                 | 1.0     |
| Increased sweating   |   |                           |         |                                     |                                |         |
| Any  | 8.9% (6.6-11.7)                                 | 7.1% (4.2-10.9)           | 0.38    | 5.0% (2.6-8.6)                      | 7.6% (4.5-11.7)                | 0.25    |
| At least one systemic reaction   |   |                           |         |                                     |                                |         |
| Any  | 50.3% (45.9-54.7)                               | 52.9% (46.6-59.2)         | 0.49    | 42.9% (36.6-49.4)                   | 34.5% (28.4-40.9)              | 0.058   |
| Severe   | 10.4% (7.9-13.4)                                | 5.5% (3.0-9.0)            | 0.022   | 8.3% (5.2-12.6)                     | 5.5% (2.9-9.2)                 | 0.22    |
| Data are incidence with 95% CI in parentheses. Any reaction means all cases, irrespective of intensity.  |   |                           |         |                                     |                                |         |
| <b>Table 5: Solicited local and systemic reactions during the first week after the first dose of vaccine and the second dose of vaccine or placebo in pregnant women living with HIV</b> |   |                           |         |                                     |                                |         |

improve the immune response in the current cohort of women. These results are in accordance with previous studies in non-pregnant women and men with HIV who had higher haemagglutination-inhibition antibody responses after high dose of inactivated influenza vaccine than after a single standard dose, but the responses were still inferior compared with those in adults without HIV.<sup>11,12</sup> Furthermore, a second dose of vaccine did not consistently increase seroresponses in pregnant or non-pregnant individuals living with HIV.<sup>11,13–15,18</sup>

Safety and immunogenicity of adjuvanted pandemic A/H1N1 vaccines have not been assessed in pregnant women with HIV, but immune responses in men and non-pregnant women living with HIV were higher with adjuvanted pandemic vaccines than with unadjuvanted pandemic vaccines.<sup>14,17</sup> A systematic review assessing the immunogenicity of different influenza vaccine strategies among individuals living with HIV found that, for the A/H1N1 strains, booster doses of adjuvanted vaccines containing 7.5 µg of antigen and 60 µg of single-dose vaccine (high-dose inactivated influenza vaccine) strategies induced higher rates of seroconversion and seroprotection than did standard doses.<sup>23</sup> The 60 µg single-dose vaccine also emerged as the most immunogenic formulation for influenza-B strains, although no single strategy was significantly more immunogenic than the standard dose for A/H3N2.<sup>23</sup> To our knowledge, only one study to date has assessed immune response to two doses of a high dose (30 µg per dose) A/H1N1 monovalent vaccine in pregnant women living with HIV.<sup>18</sup> In that report, the second high-dose injection only slightly improved the proportion of women with haemagglutination-inhibition antibody titres of 1/40 or higher (73% after the first dose and 80% after the second dose) and who seroconverted (66% after the first dose and 72% after the second dose).<sup>18</sup>

Although our cohort was heterogeneous, the participants had weak haemagglutination-inhibition antibody immune responses after vaccination with the inactivated influenza vaccine despite having generally normal CD4 cell counts and a high proportion being on antiretroviral therapy (ART). Possible reasons for this observation include residual immune dysregulation affecting both T-cell and B-cell quantities and function, immune activation, and immunosenescence.<sup>24,25</sup> Richardson and Weinberg<sup>10</sup> showed that regulatory T cells have an important role in attenuating immune responses to inactivated influenza vaccine in pregnant women living with HIV, with high proportions of influenza-specific regulatory T cells being associated with both reduced haemagglutination-inhibition antibody titres and influenza-specific cell-mediated responses.

In addition to protecting the mothers against infection with influenza virus, another potential benefit of inactivated influenza vaccine during pregnancy is to prevent infection in the neonates.<sup>5</sup> Although our

study was not powered to detect vaccine efficacy in infants exposed to HIV the incidence of influenza was similar in the three study groups. In our previous randomised controlled trial<sup>5</sup> infants born to mothers with HIV who had been given inactivated influenza vaccine had significantly higher haemagglutination-inhibition antibody titres than those born to mothers who had been given placebo, but titres were lower than in infants born to women without HIV who were given inactivated influenza vaccine. Compared with infants in the single-dose group, those in the double-dose group had increased haemagglutination-inhibition titres only for A/H1N1pdm09, but the titres and the proportion of infants with titres of 1/40 or higher against A/H1N1pdm09 were still lower than previously described for infants born to mothers without HIV who had been given a single dose of inactivated influenza vaccine (median geometric mean titre ranged from 41.8 to 93.3 and proportion with haemagglutination-inhibition titres of 1/40 or higher ranged from 60.0% to 81.1% for the three strains).<sup>5</sup> In the current study, we only did haemagglutination-inhibition assays in blood samples collected from infants in the single-dose and double-dose groups. The decision to forego the comparison of haemagglutination-inhibition titres at birth between infants exposed to single-dose and two-single-doses of inactivated influenza vaccine was based on the lack of benefit of the second dose of inactivated influenza vaccine on maternal haemagglutination-inhibition titres. Furthermore, we assumed that 28 days from time of vaccination to birth was the ideal interval for efficient transplacental transfer of antibodies,<sup>26</sup> and almost 20% of infants were born before this interval.

The three vaccination regimens were well tolerated among pregnant women, with fewer than 48% of participants having at least one solicited local injection-site reaction and fewer than 53% having at least one systemic reaction. Except for a higher incidence of local reactions in women in the double-dose group, the safety profile was similar in the double-dose and two-single-doses groups compared with the single-dose group. Four fetal deaths occurred within 14 days of receipt of inactivated influenza vaccine and were attributed as being possibly related to the vaccination only due to the temporal association because no reactogenicity was reported in these mothers after vaccination. The incidence of fetal deaths in the current study—ie, 26 per 1000 births, is similar to the incidence of stillbirths estimated in 2015 in Soweto with administrative databases (22.5 stillbirths per 1000 births).<sup>27</sup> None of the infant deaths were attributed to maternal inactivated influenza vaccine vaccination.

Limitations of this report include that the study was done during the transition of prevention of mother-to-child HIV transmission management to option B+, such that some women with CD4 counts of 350 cells per µL or higher were still given zidovudine monotherapy during pregnancy. This fact raises the

question of whether differential use of ART might influence antibody responses to vaccination. However, we have previously reported in a study of single-dose inactivated influenza vaccine in pregnant women before option B+, that plasma HIV viral load and use of ART at the time of vaccination were not associated with seroconversion 1 month after vaccination.<sup>6</sup> Similarly, other studies in people with HIV before three-drug ART was available also showed that the CD4 count threshold below which antibody responses to inactivated influenza vaccine were compromised was 200 cells per  $\mu\text{L}$ .<sup>7,28</sup> Women in this category were treated with three-drug ART in the current study. Collectively, these observations support the hypothesis that the effect of ART on antibody responses to the vaccine depends on the extent to which they promote immune reconstitution and the use of three-drug ART in the most immune-compromised women in the current study was more likely to have homogenised antibody responses to vaccination. Other study limitations were that only one influenza season was covered and a single inactivated influenza vaccine formulation was assessed at a single study site. Also, only 37% of participants had CD4 counts of less than 350 cells per  $\mu\text{L}$ . Although this low proportion prevents us from generalising our findings to a population with severe immunosuppression, it accurately represents the current landscape of HIV infection globally, in which individuals with HIV are treated earlier than in the past. By contrast, since only 27% of the women were virologically suppressed, a more in-depth analysis is needed to better understand the association between HIV viral load and immune responses to inactivated influenza vaccine. Another limitation was that only 397 (76%) of 525 infants were included in the analysis of haemagglutination-inhibition antibody titres on or before age 7 days, although they were distributed equally across study groups. Furthermore, the observed immunogenicity in the single-dose group was lower than anticipated in the sample size calculation, which could have affected the power of the study.

Despite lower humoral responses to influenza vaccination in individuals living with HIV, inactivated influenza vaccine is efficacious, and vaccination of this vulnerable population should continue.<sup>5,29</sup> Nonetheless, the results for the current study show that doubling the dose of antigen or administration of a second dose of non-adjuvanted inactivated influenza vaccine did not increase the immunogenicity of the vaccine in women living with HIV to levels similar to those of women without HIV. These findings emphasise the need for continued efforts to find an improved influenza vaccine or vaccination strategy for this population to improve protection of their infants, such as exploring the use of high-dose inactivated influenza vaccine that is currently approved for older adults in pregnant women with HIV.<sup>30</sup>

#### Contributors

MCN contributed to study conception, project supervision, data analysis, data interpretation, and wrote the first draft of the manuscript. CLC contributed to study conception, project supervision, data interpretation, and manuscript revision. AM contributed to management of the project, sample analysis, and manuscript revision. SJ contributed to management of study participants, sample and data collection, and manuscript revision. JRO, KMN, KPK, and EAFS contributed to data interpretation and manuscript revision. AW contributed to study conception, project management, data interpretation, and manuscript revision. SAM contributed to study conception, project supervision, data interpretation, and writing.

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#### Declaration of interests

MCN has received grant support from MedImmune and honoraria from Pfizer and Sanofi Pasteur outside of the submitted work. CLC has received grant support from the Bill & Melinda Gates Foundation, Pfizer, and IMPRINT, and has received honoraria from Pfizer outside of the submitted work. AW has received grant support from the Bill & Melinda Gates Foundation under the current project; grant support from GlaxoSmithKline, MedImmune, and the US National Institutes of Health outside of the submitted work. SAM has received grant support from the Bill & Melinda Gates Foundation under the current project, and grant support from Pfizer, GlaxoSmithKline, Minervax, and Novavax and personal fees from Bill & Melinda Gates Foundation outside of the submitted work. All other authors declare no competing interests.

#### Data sharing

De-identified individual participant data that underlie the results reported in this Article (text, tables, figures, and appendices) will be shared upon request. The data dictionary and study protocol are also available. Data will be available from 9 months after publication. Researchers who wish to use the data to address any specific questions not directly addressed under the study objectives and that the data would lend itself to and who provide a methodologically sound proposal that has been approved by an independent review committee can request the data. Proposals should be directed to nunesm@mpmu.co.za or madhis@mpmu.co.za. To gain access, data requestors will need to sign a data access agreement and any transfer of data will be governed by the terms of the local Ethics Committee. Data sharing will be done on a collaborative basis, with the study principal investigator (or their nominee) being included in any further interrogation of the data. The data will be provided in the format in which it has been entered at Respiratory and Meningeal Pathogens Research Unit with the necessary data dictionary.

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#### References

- Phadke VK, Omer SB. Maternal vaccination for the prevention of influenza: current status and hopes for the future. *Expert Rev Vaccines* 2016; **15**: 1255–80.
- Zaman K, Roy E, Arifeen SE, et al. Effectiveness of maternal influenza immunization in mothers and infants. *N Engl J Med* 2008; **359**: 1555–64.
- Tapia MD, Sow SO, Tamboura B, et al. Maternal immunisation with trivalent inactivated influenza vaccine for prevention of influenza in infants in Mali: a prospective, active-controlled, observer-blind, randomised phase 4 trial. *Lancet Infect Dis* 2016; **16**: 1026–35.
- Steinhoff MC, Katz J, Englund JA, et al. Year-round influenza immunisation during pregnancy in Nepal: a phase 4, randomised, placebo-controlled trial. *Lancet Infect Dis* 2017; **17**: 981–89.
- Madhi SA, Cutland CL, Kuwanda L, et al. Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med* 2014; **371**: 918–31.
- Nunes MC, Cutland CL, Dighero B, et al. Kinetics of hemagglutination-inhibiting antibodies following maternal influenza vaccination among mothers with and those without HIV infection and their infants. *J Infect Dis* 2015; **212**: 1976–87.
- Zanetti AR, Amendola A, Besana S, Boschini A, Tanzi E. Safety and immunogenicity of influenza vaccination in individuals infected with HIV. *Vaccine* 2002; **20** (suppl 5): B29–32.
- Kroon FP, Rimmelzwaan GF, Roos MT, et al. Restored humoral immune response to influenza vaccination in HIV-infected adults treated with highly active antiretroviral therapy. *AIDS* 1998; **12**: F217–23.
- Durando P, Fenoglio D, Boschini A, et al. Safety and immunogenicity of two influenza virus subunit vaccines, with or without MF59 adjuvant, administered to human immunodeficiency virus type 1-seropositive and -seronegative adults. *Clin Vaccine Immunol* 2008; **15**: 253–59.
- Richardson K, Weinberg A. Reduced immunogenicity of influenza vaccines in HIV-infected compared with uninfected pregnant women is associated with regulatory T cells. *AIDS* 2011; **25**: 595–602.
- El Sahly HM, Davis C, Kotloff K, et al. Higher antigen content improves the immune response to 2009 H1N1 influenza vaccine in HIV-infected adults: a randomized clinical trial. *J Infect Dis* 2012; **205**: 703–12.
- McKittrick N, Frank I, Jacobson JM, et al. Improved immunogenicity with high-dose seasonal influenza vaccine in HIV-infected persons: a single-center, parallel, randomized trial. *Ann Intern Med* 2013; **158**: 19–26.
- Lagler H, Grabmeier-Pfistershammer K, Touzeau-Römer V, et al. Immunogenicity and tolerability after two doses of non-adjuvanted, whole-virion pandemic influenza A (H1N1) vaccine in HIV-infected individuals. *PLoS One* 2012; **7**: e36773.
- Launay O, Desaint C, Durier C, et al. Safety and immunogenicity of a monovalent 2009 influenza A/H1N1v vaccine adjuvanted with AS03A or unadjuvanted in HIV-infected adults: a randomized, controlled trial. *J Infect Dis* 2011; **204**: 124–34.
- Cooper C, Thorne A, Klein M, et al. Immunogenicity is not improved by increased antigen dose or booster dosing of seasonal influenza vaccine in a randomized trial of HIV infected adults. *PLoS One* 2011; **6**: e17758.
- Cooper C, Klein M, Walmsley S, et al. High-level immunogenicity is achieved vaccine with adjuvanted pandemic H1N1(2009) and improved with booster dosing in a randomized trial of HIV-infected adults. *HIV Clin Trials* 2012; **13**: 23–32.
- Soonawala D, Rimmelzwaan GF, Gelinck LB, Visser LG, Kroon FP. Response to 2009 pandemic influenza A (H1N1) vaccine in HIV-infected patients and the influence of prior seasonal influenza vaccination. *PLoS One* 2011; **6**: e16496.
- Abzug MJ, Nachman SA, Muresan P, et al. Safety and immunogenicity of 2009 pH1N1 vaccination in HIV-infected pregnant women. *Clin Infect Dis* 2013; **56**: 1488–97.
- National Department of Health. The 2012 national antenatal sentinel HIV and herpes simplex type-2 prevalence survey, South Africa. Pretoria: National Department of Health, 2013.
- National Institute for Communicable Diseases monthly surveillance report. Report for 1 January to 31 January 2014. National Institute for Communicable Diseases, Division of the National Health Laboratory Services, 2014. <http://www.nicd.ac.za/assets/files/Monthly%20NICD%20Surveillance%20Report%20-%20January%202014.pdf> (accessed Oct 29, 2019).
- Weinberg A, Song IY, Walker R, et al. Anti-influenza serum and mucosal antibody responses after administration of live attenuated or inactivated influenza vaccines to HIV-infected children. *J Acquir Immune Defic Syndr* 2010; **55**: 189–96.
- Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)—a metadata-driven methodology and workflow process for providing translational research informatics support. *J Biomed Inform* 2009; **42**: 377–81.
- Zhang W, Sun H, Atiqzaman M, Sou J, Anis AH, Cooper C. Influenza vaccination for HIV-positive people: systematic review and network meta-analysis. *Vaccine* 2018; **36**: 4077–86.
- Effros RB, Fletcher CV, Gebo K, et al. Aging and infectious diseases: workshop on HIV infection and aging: what is known and future research directions. *Clin Infect Dis* 2008; **47**: 542–53.
- Lange CG, Lederman MM, Medvik K, et al. Nadir CD4+ T-cell count and numbers of CD28+ CD4+ T-cells predict functional responses to immunizations in chronic HIV-1 infection. *AIDS* 2003; **17**: 2015–23.
- Blanchard-Rohner G, Meier S, Bel M, et al. Influenza vaccination given at least 2 weeks before delivery to pregnant women facilitates transmission of seroprotective influenza-specific antibodies to the newborn. *Pediatr Infect Dis J* 2013; **32**: 1374–80.
- Madhi SA, Briner C, Maswime S, et al. Causes of stillbirths among women from South Africa: a prospective, observational study. *Lancet Glob Health* 2019; **7**: e503–12.
- Malaspina A, Moir S, Orsega SM, et al. Compromised B cell responses to influenza vaccination in HIV-infected individuals. *J Infect Dis* 2005; **191**: 1442–50.
- Madhi SA, Maskew M, Koen A, et al. Trivalent inactivated influenza vaccine in African adults infected with human immunodeficient virus: double blind, randomized clinical trial of efficacy, immunogenicity, and safety. *Clin Infect Dis* 2011; **52**: 128–37.
- Grohskopf LA, Sokolow LZ, Broder KR, Walter EB, Fry AM, Jernigan DB. Prevention and control of seasonal influenza with vaccines: recommendations of the Advisory Committee on Immunization Practices—United States, 2018–19 Influenza Season. *MMWR Recomm Rep* 2018; **67**: 1–20.

# Effectiveness of Influenza Vaccination of Pregnant Women for Prevention of Maternal and Early Infant Influenza-Associated Hospitalizations in South Africa: A Prospective Test-Negative Study

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**Background.** Influenza vaccination during pregnancy reduces influenza-associated illness in the women and their infants, but effectiveness estimates against influenza-associated hospitalization are limited and lacking from settings with high human immunodeficiency virus (HIV) infection prevalence. We assessed the effect of maternal vaccination in HIV-uninfected women and women with HIV in preventing influenza-associated hospitalizations in infants and the women.

**Methods.** During 2015–2018, influenza vaccination campaigns targeting pregnant women were augmented at selected antenatal clinics; these were coupled with prospective hospital-based surveillance for acute respiratory or febrile illness in infants aged <6 months and cardiorespiratory illness among pregnant or postpartum women. Vaccine effectiveness (VE) was assessed using a test-negative case-control study.

**Results.** Overall, 71 influenza-positive and 371 influenza-negative infants were included in the analysis; mothers of 26.8% of influenza-positive infants were vaccinated during pregnancy compared with 35.6% of influenza-negative infants, corresponding to an adjusted VE (aVE) of 29.0% (95% confidence interval [CI], –33.6% to 62.3%). When limited to vaccine-matched strains, aVE was 65.2% (95% CI, 11.7%–86.3%). For maternal hospitalizations, 56 influenza-positive and 345 influenza-negative women were included in the analysis, with 28.6% of influenza-positive women being vaccinated compared with 38.3% of influenza-negatives, for an aVE of 46.9% (95% CI, –2.8% to 72.5%). Analysis restricted to HIV-uninfected women resulted in 82.8% (95% CI, 40.7%–95.0%) aVE. No significant aVE (–32.5% [95% CI, –208.7% to 43.1%]) was detected among women with HIV.

**Conclusions.** Influenza vaccination during pregnancy prevented influenza-associated hospitalizations among young infants when infected with vaccine strains and among HIV-uninfected women.

**Keywords.** influenza; pregnancy; vaccine.

Randomized controlled trials (RCTs) performed in low- and middle-income countries (LMICs) have demonstrated that the

efficacy of seasonal influenza vaccination during pregnancy is 50%–70% in preventing mild to moderately severe laboratory-confirmed influenza-associated illness in pregnant women [1, 2]. After vaccination, maternal antibodies cross the placenta and may offer protection to the infant during the first months of life. Therefore, an additional benefit of maternal vaccination has been the protection of the young infants. Maternal vaccination to protect young infants is pertinent since there is no influenza vaccine approved for use in infants aged <6 months. A meta-analysis of 4 RCTs in pregnant women revealed a combined vaccine efficacy of 36% (95% confidence interval [CI], 22%–48%) against laboratory-confirmed influenza-associated illness in infants aged <6 months [1–5]. Vaccine efficacy was higher if the analyses were restricted to the first 8 weeks (86%) or 4 months (68%) of life [2, 6].

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From the previous RCTs, only a South African trial assessed vaccine immunogenicity and efficacy among pregnant women living with human immunodeficiency virus (WLWH) and their infants [1]. In that study, despite WLWH having lower levels of neutralization, hemagglutination inhibition, and H1-hemagglutinin stalk domain antibodies following vaccination compared with those without human immunodeficiency virus (HIV) [7–9], the overall vaccine efficacy against influenza-associated illness was similar in both groups (57.7% and 50.4%, respectively) [1]. The study in WLWH was not powered to detect vaccine efficacy in the HIV-exposed infants; nevertheless, similar influenza attack rates were detected in infants born to WLWH in the vaccinated (5.0%) or placebo (6.8%) groups. Attack rates were lower in infants of HIV-uninfected placebo recipients (3.6%) [1].

The low incidence of influenza-associated hospitalization within each influenza season poses a challenge to demonstrate the effectiveness of influenza vaccination against severe disease. Nonetheless, a few observational studies, mainly from Europe and the United States (US), have reported a 45%–92% reduction of laboratory-confirmed influenza-associated hospitalizations in infants aged <6 months by vaccinating pregnant women [3, 10–15]. Furthermore, a multicountry study conducted in Australia, Canada, Israel, and the US reported a vaccine effectiveness (VE) of 40% (95% CI, 12%–59%) against influenza-associated hospitalizations in pregnant women from 2010 to 2016 [16].

Understanding the effect of influenza vaccination on severe influenza outcomes in infants and during pregnancy in LMICs, including among WLWH and their infants, will help to delineate the potential benefit of the maternal immunization programs in these countries. We conducted a prospective test-negative study over 4 consecutive influenza seasons. The primary objective was to evaluate the effectiveness of influenza vaccination of pregnant women in reducing the risk of polymerase chain reaction (PCR)-confirmed influenza-associated hospitalization in their infants during the first 6 months of life, and to estimate VE in pregnant or postpartum women.

## METHODS

From 2015 to 2018, once the inactivated influenza vaccine (IIV) formulated for the Southern Hemisphere was available in South Africa, we provided additional IIV doses to selected antenatal clinics in 2 South African cities (Johannesburg and Cape Town), aiming to increase vaccination coverage of pregnant women attending those clinics to at least 50% [17]. Study staff based at the clinics compiled vaccination registers of the women attending the clinics during the period that IIV was available. Vaccination was also documented at the discretion of the attending nurse on the individual maternal antenatal cards. Prospective active surveillance was conducted among infants aged <6 months hospitalized for acute respiratory or febrile illness

(ARI/FI), from when IIV was available to the end of each calendar year, at 4 hospitals in the 2 cities; and among pregnant women and women within 42 days postdelivery hospitalized for cardiorespiratory illness in 6 hospitals during the influenza seasons. More details on study design and population are shown in the [Supplementary Material](#). Enrolled participants had respiratory swabs collected and tested by real-time PCR for influenza as previously described [1, 18]. Influenza A-positive samples were subtyped as A(H1N1)pdm09 or A(H3N2). All A(H1N1)pdm09 viruses were considered to be vaccine-matched as they belonged to the 6B lineage with antigenic characteristics similar to vaccine strains; for A(H3N2) each year we compared the dominant lineage circulating in South Africa with the vaccine lineage and considered them matched or unmatched ([Supplementary Table 1](#)) [19–23]. Influenza B-positive samples were typed as Victoria or Yamagata lineages and compared with the vaccine lineages ([Supplementary Table 1](#)).

## Influenza Vaccination

During the study period, only trivalent IIV formulations were available. The virus strains contained in the vaccines recommended for the Southern Hemisphere for each year are shown in [Supplementary Table 1](#). Maternal immunization status was determined based on the information in the antenatal cards, if these were available at the time of admission, or study vaccination registers [17]. Women were considered vaccinated if there was written documentation of receipt of IIV during pregnancy and if they were vaccinated  $\geq 14$  days before giving birth (infant analysis) or  $\geq 14$  days before hospital admission (maternal analysis). Women vaccinated <14 days before birth/admission and those who had unknown vaccination status or had a missing vaccination date were excluded from analyses.

## Statistical Analysis

The primary objective of the study was to estimate VE against influenza-associated hospitalization in infants aged <6 months. Assuming 5% significance level, 80% power, case-control ratio ranging from 1:4 to 1:8, vaccine coverage of 50%, and a VE of 60%, we estimated a minimum sample size of 46 cases.

Two unmatched test-negative case-control analyses for infants and women were used to estimate VE: (1) For infants, cases were infants hospitalized with ARI/FI, whose mothers were eligible to have received IIV during pregnancy, aged <6 months at hospital admission with PCR-confirmed influenza; for each infant case, 5 controls were randomly selected among the influenza-negative hospitalized infants with ARI/FI enrolled during the months that cases were also enrolled; (2) in the women, those aged  $\geq 18$  years, hospitalized for cardiorespiratory illness during pregnancy or 42 days postpartum, who would have been eligible to receive IIV during pregnancy were enrolled; cases were those with PCR-confirmed influenza, and controls those who tested negative.

**Table 1. Characteristics of Hospitalized Infants Born to Mothers With Known Influenza Vaccination Status Who Tested Influenza Polymerase Chain Reaction Positive and Randomly Selected Control Infants Testing Influenza Negative—South Africa, 2015–2018**

| Characteristic   | Influenza-Positive Cases (n = 71) | Influenza-Negative Controls (n = 371) | P Value |
|--|-----------------------------------|---------------------------------------|---------|
| <b>Age at admission</b>  |                                   |                                       |         |
| Mean age, d (SD)   | 66.2 (38.4)                       | 46.2 (37.2)                           | <.001   |
| <3 mo  | 54 (76.1)                         | 316 (85.2)                            | .057    |
| 3–5 mo   | 17 (23.9)                         | 55 (14.8)                             |         |
| <b>HIV exposure</b>  |                                   |                                       |         |
| Exposed  | 16/70 (22.9)                      | 67 (18.1)                             | .35     |
| Unexposed  | 54/70 (77.1)                      | 304 (81.9)                            |         |
| <b>Sex</b>   |                                   |                                       |         |
| Male   | 41 (57.8)                         | 206 (55.5)                            | .73     |
| Female   | 30 (42.3)                         | 165 (44.5)                            |         |
| <b>Race</b>  |                                   |                                       |         |
| Black African  | 50 (70.4)                         | 269 (72.5)                            | .94     |
| South African Coloured   | 19 (26.8)                         | 89 (24.0)                             |         |
| White  | 1 (1.4)                           | 8 (2.2)                               |         |
| Asian  | 1 (1.4)                           | 5 (1.4)                               |         |
| <b>Term (<math>\geq 37</math> wk gestation) or normal birthweight (<math>\geq 2500</math> g)</b> |                                   |                                       |         |
| Yes  | 57 (80.3)                         | 318 (85.7)                            | .24     |
| No   | 14 (19.7)                         | 53 (14.3)                             |         |
| <b>Ever breastfed</b>  |                                   |                                       |         |
| Yes  | 54/69 (78.3)                      | 301/364 (82.7)                        | .38     |
| No   | 15/69 (21.7)                      | 63/364 (17.3)                         |         |
| <b>Study site</b>  |                                   |                                       |         |
| Chris Hani Baragwanath Academic Hospital, Johannesburg   | 26 (36.6)                         | 159 (42.9)                            | .74     |
| Rahima Moosa Mother and Child Hospital, Johannesburg   | 17 (23.9)                         | 77 (20.8)                             |         |
| Red Cross Hospital, Cape Town  | 26 (36.6)                         | 121 (32.6)                            |         |
| Mitchell's Plain Hospital, Cape Town   | 2 (2.8)                           | 14 (3.8)                              |         |
| <b>Antenatal clinic attended supplemented with vaccine</b>                                       |                                   |                                       |         |
| Yes  | 42/64 (65.6)                      | 214/348 (61.5)                        | .53     |
| No   | 22/64 (34.4)                      | 134/348 (38.5)                        |         |
| <b>Year of enrollment</b>  |                                   |                                       |         |
| 2015   | 12 (16.9)                         | 49 (13.2)                             | .58     |
| 2016   | 24 (33.8)                         | 111 (29.9)                            |         |
| 2017   | 14 (19.7)                         | 98 (26.4)                             |         |
| 2018   | 21 (29.6)                         | 113 (30.5)                            |         |
| <b>Period of influenza season</b>  |                                   |                                       |         |
| Early  | 14 (19.7)                         | 66 (17.8)                             | .46     |
| Middle   | 20 (28.2)                         | 118 (31.8)                            |         |
| Late   | 28 (39.4)                         | 129 (34.8)                            |         |
| Outside  | 9 (12.7)                          | 58 (15.6)                             |         |
| <b>Influenza vaccination during pregnancy (<math>&gt;13</math> d before delivery)</b>            |                                   |                                       |         |
| Yes  | 19 (26.8)                         | 132 (35.6)                            | .15     |
| No   | 52 (73.2)                         | 239 (64.4)                            |         |

Values are presented as No. (%) unless stated otherwise. The number of participants with available information is indicated (no./No.) if different from the total number of participants. Abbreviations: HIV, human immunodeficiency virus; SD, standard deviation.

VE was calculated as  $1 - \text{odds ratio} [\text{ratio of odds of vaccination among cases to the odds of vaccination among controls}] \times 100\%$  using logistic regression. Both VE estimates were adjusted for HIV infection (maternal analysis) or HIV exposure (infant analysis) and any other variables that were significantly associated with the exposure (vaccination) or the outcome (influenza infection) on univariate analysis. Other potential confounder variables were evaluated by assessing if they altered the VE estimates; however, none changed the adjusted VE by  $>10\%$  and

thus were not included in the final model. A variable was created to define influenza season timing at hospital admission by dividing each influenza season into early, middle, and late season terciles of equal duration; for the infant analysis, an “outside of season” period was also considered. The South African influenza seasons were defined using data from the influenza sentinel surveillance from the National Institute for Communicable Diseases [24]. Descriptive statistics were used to characterize cases and controls and the proportion of women who received IIV.

**Table 2. Effectiveness of Influenza Vaccine Administered in Pregnancy Against Influenza-Confirmed Hospitalization in Infants <6 Months of Age—South Africa, 2015–2018**

| Characteristic                       | Influenza Positive,<br>No. Vaccinated/Total No. | Influenza Negative,<br>No. Vaccinated/Total No. | Unadjusted VE, %<br>(95% CI) | Adjusted VE, %<br>(95% CI) <sup>a</sup> |
|--------------------------------------|---|---|------------------------------|---|
| <b>All infants</b>                   |   |   |                              |   |
| Overall                              | 19/71   | 132/371   | 33.8 (–16.6 to 62.5)         | 29.0 (–33.6 to 62.3)                    |
| Born at term                         | 16/57   | 122/318   | 37.3 (–16.6 to 66.3)         | 38.4 (–23.5 to 69.3)                    |
| Admitted to hospital at <3 mo of age | 14/54   | 120/316   | 42.8 (–9.5 to 70.1)          | 42.8 (–17.0 to 72.0)                    |
| Vaccine-matched strains              | 7/43  | 132/371   | <b>64.8 (18.7–84.8)</b>      | <b>65.2 (11.7–86.3)</b>                 |
| Born at term                         | 6/35  | 122/318   | <b>66.8 (17.6–86.6)</b>      | <b>69.3 (15.1–88.9)</b>                 |
| Admitted to hospital at <3 mo of age | 6/36  | 120/316   | <b>67.3 (19.2–86.8)</b>      | <b>70.6 (21.0–89.1)</b>                 |
| <b>HIV unexposed</b>                 |   |   |                              |   |
| Overall                              | 15/54   | 111/304   | 33.1 (–26.8 to 64.7)         | 24.1 (–55.1 to 62.8)                    |
| Born at term                         | 12/41   | 104/267   | 35.1 (–32.7 to 68.3)         | 34.6 (–45.9 to 70.6)                    |
| Admitted to hospital at <3 mo of age | 10/39   | 100/259   | 45.2 (–17.4 to 74.4)         | 43.8 (–29.3 to 75.6)                    |
| Vaccine-matched strains              | 6/31  | 111/304   | 58.3 (–4.8 to 83.4)          | 50.6 (–39.6 to 82.5)                    |
| Born at term                         | 5/23  | 104/267   | 56.5 (–20.8 to 84.3)         | 55.8 (–35.2 to 85.6)                    |
| Admitted to hospital at <3 mo of age | 5/26  | 100/259   | 62.1 (–3.6 to 86.2)          | 62.2 (–10.7 to 87.1)                    |
| <b>HIV exposed</b>                   |   |   |                              |   |
| Overall                              | 4/16  | 21/67   | 27.0 (–153.3 to 78.9)        | 28.7 (–226.4 to 84.4)                   |
| Born at term                         | 4/15  | 18/51   | 33.3 (–139.9 to 81.5)        | 58.5 (–125.6 to 92.4)                   |
| Admitted to hospital at <3 mo of age | 4/14  | 20/57   | 26.0 (–166.4 to 79.4)        | 26.7 (–232.5 to 83.8)                   |
| Vaccine-matched strains              | 1/11  | 21/67   | 78.1 (–82.4 to 97.4)         | 87.9 (–48.5 to 99.0)                    |
| Born at term                         | 1/11  | 18/51   | 81.7 (–54.9 to 97.8)         | <b>96.1 (10.0–99.8)</b>                 |
| Admitted to hospital at <3 mo of age | 1/9   | 20/57   | 76.9 (–98.3 to 97.3)         | 86.9 (–61.7 to 98.9)                    |

Values in bold denote significant vaccine effectiveness estimates. Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; VE, vaccine effectiveness.

<sup>a</sup>Vaccine effectiveness overall adjusted for year of study, hospital, period of influenza season when hospitalization occurred, age at hospitalization, antenatal clinic attended by the mother being supplemented with vaccine (yes or no), born at term (yes or no), and HIV exposure status.

In the primary infant analysis, VE was estimated for hospitalizations associated with any PCR-confirmed influenza illness from birth until 6 months of age. Subgroup analyses were conducted by HIV infection status of the mother (ie, HIV exposure), restricted to infants born at term (defined as infants born at  $\geq 37$  weeks' gestation or with a birthweight  $\geq 2500$  g) or to infants admitted at <3 months of age. For the maternal analysis, VE was estimated for hospitalizations occurring during the influenza seasons associated with any PCR-confirmed influenza illness at any stage during pregnancy and up until 42 days postpartum; subgroup analyses were conducted by HIV infection status. Both infant and maternal analyses were also done to estimate VE limited to viruses matched with the vaccine strains for each year. Odds ratios were considered statistically significant when the 95% CIs did not overlap 1.0. Analyses were performed with Stata software, version 13.1 (StataCorp, College Station, Texas).

#### Patient Consent Statement

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (M140826) and University of Cape Town (835/2014) and conducted in accordance with Good Clinical Practice guidelines. The Centers for Disease Control and Prevention Institutional Review Board (IRB) relied on the local IRB review (#6746, 45

Code of Federal Regulations [CFR] part 46; 21 CFR part 56). Written informed consent was obtained from the infants' legal guardian or the hospitalized women.

## RESULTS

Each year, the vaccination of pregnant women started as soon as IIV was available in the country. The timing of vaccine availability was 22 April–31 July 2015; 4 April–16 August 2016; 3 April–16 August 2017; and 13 March–29 June 2018.

#### Infant Analyses

Overall, 3484 infants aged <6 months born to women eligible to have received IIV during pregnancy and who were born at least 14 days after vaccines were available were hospitalized with ARI/FI at the participating hospitals and enrolled in the study. All the infants were tested by PCR for influenza virus, of whom 83 (2.4%) had a positive result. Maternal vaccination status was unavailable for 3 of the 83 (3.6%) infants and, for another 9 (10.8%), their mothers were vaccinated <14 days before delivery. Among all the influenza-negative infants enrolled, 421 were randomly selected, of whom 25 (5.9%) were excluded because maternal vaccination status was unavailable and 25 because their mothers were vaccinated <14 days before delivery. Accordingly, 71 influenza-positive and 371 influenza-

negative infants were included in the analysis (Supplementary Figure 1).

Infant cases were older than controls (mean age, 66.2 vs 46.2 days;  $P < .001$ ), whereas all other demographic characteristics were similar between the 2 groups (Table 1).

Overall, 151 (34.3%) infants were born to vaccinated mothers, and 291 (65.7%) were born to unvaccinated mothers. Infants born to vaccinated mothers compared with unvaccinated, were younger at hospital admission, and a higher percentage were born at term. Furthermore, a higher percentage of vaccinated mothers attended antenatal care at the clinics where vaccines were supplied by the study. Other significant differences between the vaccinated and unvaccinated group were year of enrollment and timing during the influenza season that hospitalization occurred (Supplementary Table 2).

Across study seasons and sites, 26.8% (19/71) of mothers of the infant cases were vaccinated during pregnancy compared with 35.6% (132/371) of the control infants, yielding an adjusted VE (aVE) of 29.0% (95% CI, -33.6% to 62.3%). Similar non-significant aVE point estimates were obtained when restricting the analysis to infants <3 months old or to infants born at term. When only vaccine-matched strains across the different years were included in the analysis (ie, excluding 4 influenza B un-subtyped cases, 18 influenza B/Victoria, and 6 influenza B/Yamagata), aVE was 65.2% (95% CI, 11.7%–86.3%), with similar VE in infants <3 months old or born at term (Table 2).

Among HIV-unexposed infants, the maternal vaccination coverage was 27.8% in the cases and 36.8% in controls, corresponding to an aVE of 24.1% (95% CI, -55.1% to 62.8%). Only 16 PCR-confirmed influenza virus infections were detected among HIV-exposed infants and 25% of their mothers were vaccinated compared to 31.4% of mothers from control infants, for an aVE of 28.7% (95% CI, -226.4% to 84.4%). Similar VE point estimates were obtained in both HIV exposure-stratified groups restricting the analyses to infants <3 months old or born at term. For only vaccine-matched strains, the aVE was 50.6% (95% CI, -39.6% to 82.5%) among HIV-unexposed infants and 87.9% (95% CI, -48.5% to 99.0%) for HIV-exposed infants (Table 2).

#### Maternal Analyses

During the study period, 415 pregnant or postpartum women eligible to have received IIV during pregnancy were hospitalized during the influenza seasons at least 14 days after IIV was available. Fifty-nine (14.2%) PCR-confirmed influenza cases were detected, among whom vaccination status was unavailable for 3 (5.1%). Among the 356 influenza-negative controls, 3 (0.8%) had unknown vaccination status, 1 (0.3%) was vaccinated but date was missing, and 7 (2.0%) received vaccine <14 days before hospitalization. Therefore, 56 cases and 345 controls were included in the maternal analysis (Supplementary Figure 2). Cases and controls had similar characteristics

(Table 3). Overall, 148 (36.9%) women received IIV before admission; vaccinated and unvaccinated women differed by admitting hospital, pregnancy trimester at admission, and year of hospitalization (Supplementary Table 3).

Overall, 28.6% of the cases were vaccinated compared with 38.3% of the controls, for an aVE of 46.9% (95% CI, -2.8% to 72.5%). When analysis was stratified by HIV infection status, HIV-uninfected women had a vaccination coverage of 10.7% among cases and 40.3% among controls, resulting in an aVE of 82.8% (95% CI, 40.7%–95.0%). In WLWH, vaccination coverage was 46.4% and 35.8% among cases and controls, respectively (aVE, -32.5% [95% CI, -208.7% to 43.1%]). Similar results were obtained when restricted to vaccine-matched strains (Table 4).

#### Circulating Strains

The percentage of influenza strains detected among study participants matched to the annual vaccines' formulations varied from 38.7% in 2018, when 2 distinct peaks of A(H1N1)pdm09 and influenza B/Victoria (vaccine mismatch) were detected in the country, to 93% in 2016 when there was a good match between influenza B and A(H3N2) co-circulating strains and the vaccine strains (Table 5).

#### DISCUSSION

In our study covering 4 consecutive influenza seasons, influenza vaccination during pregnancy had an estimated effectiveness of 65% against influenza-associated hospital admissions in young infants, although VE was only demonstrated against influenza viruses considered to be vaccine matches. Overall, 84% of all enrolled infants were aged <3 months at hospital admission, and therefore the VE point estimates were similar considering all ages or restricting to infants aged <3 months. In a stratified analysis by HIV exposure status, although the VE point estimates for vaccine-matched strains were 58% for HIV-unexposed infants and 78% for HIV-exposed infants, these were nonsignificant, due to the small number of cases that only provided power of 48% and 27%, respectively. During the study period, surveillance was also performed for influenza-associated hospitalizations among women. Among HIV-uninfected women, high VE estimates were detected either for all influenza (82%) and for vaccine-matched strains only (85%). In this study, maternal influenza vaccination did not demonstrate effectiveness against hospitalization in WLWH.

In previous RCTs over 2 influenza seasons in South Africa, the efficacy of influenza vaccination during pregnancy against any PCR-confirmed influenza illness was similar among HIV-uninfected women (50.4% [95% CI, 14.5%–71.2%]) and WLWH (57.7% [95% CI, .2%–82.1%]), notwithstanding WLWH having lower humoral immune responses following

**Table 3. Characteristics of Hospitalized Pregnant and Postpartum Women With Known Influenza Vaccination Status Who Tested Influenza Positive or Negative by Polymerase Chain Reaction—South Africa, 2015–2018**

| Characteristic  | Influenza-Positive Cases (n=56) | Influenza-Negative Controls (n=345) | P Value |
|---|---------------------------------|-------------------------------------|---------|
| <b>Age at admission, y</b>  |                                 |                                     |         |
| Mean (SD)   | 30.1 (6.6)                      | 29.6 (6.3)                          | .60     |
| 18–24   | 14 (25.0)                       | 86 (25.0)                           | .99     |
| 25–34   | 27 (48.2)                       | 167 (48.6)                          |         |
| ≥35   | 15 (26.8)                       | 91 (26.5)                           |         |
| <b>HIV infection</b>  |                                 |                                     |         |
| Infected  | 28 (50.0)                       | 148/344 (43.0)                      | .33     |
| Uninfected  | 28 (50.0)                       | 196/344 (57.0)                      |         |
| <b>Race</b>   |                                 |                                     |         |
| Black African   | 40 (71.4)                       | 267 (77.4)                          | .67     |
| South African Coloured  | 15 (26.8)                       | 69 (20.0)                           |         |
| White   | 1 (1.8)                         | 6 (1.7)                             |         |
| Asian   | 0 (0)                           | 3 (0.9)                             |         |
| <b>Pregnancy trimester at admission</b>                                 |                                 |                                     |         |
| First   | 1/55 (1.8)                      | 8/340 (2.4)                         | .54     |
| Second  | 13/55 (23.6)                    | 77/340 (22.7)                       |         |
| Third   | 40/55 (72.7)                    | 231/340 (67.9)                      |         |
| Postpartum  | 1/55 (1.7)                      | 24/340 (7.1)                        |         |
| <b>Study site</b>   |                                 |                                     |         |
| Chris Hani Baragwanath Academic Hospital, Johannesburg                  | 13 (23.2)                       | 96 (27.8)                           | .81     |
| Rahima Moosa Mother and Child Hospital, Johannesburg                    | 21 (37.5)                       | 123 (35.7)                          |         |
| Helen Joseph Hospital, Johannesburg                                     | 2 (3.6)                         | 12 (3.5)                            |         |
| Groote-Schuur Hospital, Cape Town                                       | 5 (8.9)                         | 34 (9.9)                            |         |
| Mowbray Hospital, Cape Town   | 10 (18.9)                       | 40 (11.6)                           |         |
| Mitchell's Plain Hospital, Cape Town                                    | 5 (8.9)                         | 40 (11.6)                           |         |
| <b>Comorbidity</b>  |                                 |                                     |         |
| Yes   | 13 (23.2)                       | 59 (17.1)                           | .27     |
| No  | 43 (76.8)                       | 286 (82.9)                          |         |
| <b>Smoker</b>   |                                 |                                     |         |
| Yes   | 4 (7.1)                         | 31 (9.0)                            | .80     |
| No  | 52 (92.9)                       | 314 (91.0)                          |         |
| <b>Alcohol consumption</b>  |                                 |                                     |         |
| Yes   | 2 (3.6)                         | 16 (4.6)                            | .99     |
| No  | 54 (96.4)                       | 329 (95.4)                          |         |
| <b>Period of influenza season</b>                                       |                                 |                                     |         |
| Early   | 24 (42.9)                       | 134 (38.8)                          | .72     |
| Middle  | 20 (35.7)                       | 120 (34.8)                          |         |
| Late  | 12 (21.4)                       | 91 (26.4)                           |         |
| <b>Year of enrollment</b>   |                                 |                                     |         |
| 2015  | 4 (7.1)                         | 34 (9.9)                            | .56     |
| 2016  | 19 (33.9)                       | 91 (26.4)                           |         |
| 2017  | 15 (26.8)                       | 85 (24.6)                           |         |
| 2018  | 18 (32.1)                       | 135 (39.1)                          |         |
| <b>Influenza vaccination during pregnancy &gt;13 d before admission</b> |                                 |                                     |         |
| Yes   | 16 (28.6)                       | 132 (38.3)                          | .16     |
| No  | 40 (71.4)                       | 213 (61.7)                          |         |

Values are presented as No. (%) unless stated otherwise. The number of participants with available information is indicated (no./No.) if different from the total number of participants. Abbreviations: HIV, human immunodeficiency virus; SD, standard deviation.

vaccination [7–9]. The efficacy of influenza vaccination against confirmed influenza illness was also 75.5% (95% CI, 9.2%–95.6%) among nonpregnant South African adults with HIV [25]. We are not aware of any study reporting on the efficacy of influenza vaccination specifically against influenza-associated hospitalization among people with HIV. Residual confounding

inherent to observational studies may, however, explain the differences in the current results with the RCTs.

Throughout the entire study, both influenza A and B viruses circulated in South Africa. Genetic data showed that during the study period, most of the circulating A(H1N1)pdm09 viruses were similar to the ones included in the seasonal vaccines

**Table 4. Effectiveness of Influenza Vaccine Administered in Pregnancy Against Maternal Influenza-Confirmed Hospitalization—South Africa, 2015–2018**

| Participants            | Influenza Positive,<br>No. Vaccinated/Total No. | Influenza Negative,<br>No. Vaccinated/Total No. | Unadjusted VE, %<br>(95% CI) | Adjusted VE, %<br>(95% CI) <sup>a</sup> |
|-------------------------|---|---|------------------------------|---|
| <b>All mothers</b>      |   |   |                              |   |
| Overall                 | 16/56   | 132/345   | 35.5 (–19.9 to 65.2)         | 46.9 (–2.8 to 72.5)                     |
| Vaccine-matched strains | 13/43   | 132/345   | 30.1 (–38.9 to 64.8)         | 44.0 (–17.4 to 73.3)                    |
| <b>Without HIV</b>      |   |   |                              |   |
| Overall                 | 3/28  | 79/196  | <b>82.2 (39.1–94.8)</b>      | <b>82.8 (40.7–95.0)</b>                 |
| Vaccine-matched strains | 2/22  | 79/196  | <b>85.2 (34.9–96.6)</b>      | <b>85.8 (36.7–96.8)</b>                 |
| <b>Living with HIV</b>  |   |   |                              |   |
| Overall                 | 13/28   | 53/148  | –55.3 (–251.0 to 31.2)       | –32.5 (–208.7 to 43.1)                  |
| Vaccine-matched strains | 11/21   | 53/148  | –97.2 (–394.7 to 21.4)       | –78.9 (–370.5 to 32.0)                  |

Values in bold denote significant vaccine effectiveness estimates. Abbreviations: CI, confidence interval; HIV, human immunodeficiency virus; VE, vaccine effectiveness.

<sup>a</sup>VE adjusted for year of study, hospital, pregnancy trimester or postpartum period when hospitalization occurred, and HIV infection status for all mothers.

**Table 5. Influenza Strains Detected During the Study Period in Infants and Women**

| Influenza Strain        | 2015              | 2016              | 2017              | 2018              | Overall           |
|-------------------------|-------------------|-------------------|-------------------|-------------------|-------------------|
| A(H1N1)pdm09            | <b>2</b>          | <b>12</b>         | <b>1</b>          | <b>15</b>         | 29                |
| A(H3N2)                 | 1                 | <b>12</b>         | <b>19</b>         | ...               | 32                |
| B/Victoria              | 2                 | <b>16</b>         | ...               | 24                | 42                |
| B/Yamagata              | <b>9</b>          | ...               | 9                 | ...               | 18                |
| B (Unsubtyped)          | 2                 | 3                 | ...               | ...               | 5                 |
| Vaccine-matched strains | <b>11 (68.8%)</b> | <b>40 (93.0%)</b> | <b>20 (69.0%)</b> | <b>15 (38.5%)</b> | <b>86 (67.7%)</b> |

Bolded values indicate viruses similar to the vaccine viruses.

[20–23]. For A(H3N2), using national sequencing information, for each study year but 2015, there was good concordance between the circulating and vaccine viruses. In 2015 the lineage 3C.3a was included in the vaccine, but circulating strains were in the 3C.2a lineage and therefore the A(H3N2) cases in 2015 were considered not similar to vaccine viruses. For the influenza B lineages, the viruses identified in study participants during 2017 and 2018 were not of the same lineage that was contained in the trivalent vaccine. It has been suggested that trivalent IIV offers cross-protection against nonvaccine B lineages [26, 27]; in our study, however, where most of the influenza B vaccine-unmatched viruses were detected in the infants, a protective effect in the infants was only detected for vaccine matches. This result is different from the vaccine efficacy among HIV-unexposed infants in the South African RCT, which was similar either including all PCR-confirmed influenza episodes (48.8% [95% CI, 11.6%–70.40%]) or excluding the 17 cases of nonvaccine B lineage (48.2% [95% CI, –.8% to 73.3%]) [1]. Our VE of 65% among the infants is in line with previous reports from Europe and the US where estimates of 45%–92% were reported [3, 10–15]. A study from England that measured the effectiveness of maternal influenza vaccination in preventing influenza-associated hospitalizations in infants aged <6 months over 2 consecutive seasons of 2013–2014 (dominated by A[H1N1]pdm09) and 2014–2015 (dominated by a drifted A[H3N2] strain) reported an overall VE of 64% (95% CI,

5%–87%) in 2013–2014 and 50% (95% CI, 8%–73%) in 2014–2015, with a similar estimate for 2014–2015 (58% [95% CI, 7%–81%]) if restricted to infants infected with the dominant drifted A(H3N2) strain [15].

Our VE estimates among HIV-uninfected women are higher than previously reported in a study across 4 countries from 2010 to 2016 (40% [95% CI, 12%–59%]), although in that study only 16% of all hospitalized women were vaccinated [16].

From 2015 to 2018, our study supplemented the national influenza vaccination program at selected antenatal clinics aiming at increasing vaccination rates to at least 50%. Although the vaccination campaigns were very successful and >75% of the women who received care at the selected clinics were vaccinated during the campaigns [17], the vaccination coverage among the hospitalized study participants, many of whom sought antenatal care at clinics which did not have supplemental vaccine supply, was <36%. This low vaccination coverage impacted the power of our analyses and might be the result of several factors. First, although analyses were restricted to those who were eligible to have received IIV during pregnancy, it is possible that not all participants had antenatal visits during the period that vaccines were available, even though our campaigns lasted for 3–4 months, and South African national estimates for 2016 revealed that 76% of pregnant women attended ≥4 antenatal care visits and that 47% of women had their first visit during the first pregnancy trimester [28]. Second, not all



women attended antenatal care at clinics supplemented with study vaccine and, as shown in [Supplementary Table 2](#), only 18% of the women attending other clinics were vaccinated. Third, although the vaccination campaigns aimed to deliver vaccines before the start of the influenza season, the actual timing of vaccination was determined by the availability of vaccines at the selected clinics, and as previously reported across the study years, approximately only 52% of the vaccines were administered prior to the start of the influenza seasons [17]. Vaccination campaigns aimed at reducing the burden of influenza-associated illness, especially among infants, need to consider how quickly vaccination can be implemented once vaccines are available to maximize the overlap between vaccination opportunity and the risk of influenza infection in the population.

Limitations of our study include that the vaccination coverage among study participants was lower than initially anticipated, leading to a decrease in the power of the analyses. We tried to systematically document the vaccination status of the women attending the antenatal clinics selected for vaccine supplementation, and in the analyses, women were considered vaccinated only if written evidence was available; however, women could have been vaccinated and if this information was not recorded in their antenatal cards or in the study registers, they would be misclassified as unvaccinated. This misclassification would likely have been nondifferential, which would potentially result in an underestimation of the VE. Since written informed consent was required for participation, not every eligible patient was included in the study; however, differences in participation should have been similar according to influenza infection and vaccination status. Overall, as this is an observational study, there could be residual confounding that we were unable to account for. A further limitation is that we were unable to investigate VE in WLWH stratified by their degree of immunosuppression, as these data were not available.

Our study provides additional evidence for the benefit of maternal influenza vaccination to prevent severe disease in infants and among HIV-uninfected women. The effectiveness in WLWH may depend on the degree of immunosuppression, but we were unable to assess that, and further studies are needed.

#### Supplementary Data

Supplementary materials are available at *Open Forum Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

#### Notes

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#### References

- Madhi SA, Cutland CL, Kuwanda L, et al. Influenza vaccination of pregnant women and protection of their infants. *N Engl J Med* **2014**; 371:918–31.
- Tapia MD, Sow SO, Tamboura B, et al. Maternal immunisation with trivalent inactivated influenza vaccine for prevention of influenza in infants in Mali: a prospective, active-controlled, observer-blind, randomised phase 4 trial. *Lancet Infect Dis* **2016**; 16:1026–35.
- Nunes MC, Madhi SA. Influenza vaccination during pregnancy for prevention of influenza confirmed illness in the infants: a systematic review and meta-analysis. *Hum Vaccin Immunother* **2018**; 14:758–66.
- Steinhoff MC, Katz J, Englund JA, et al. Year-round influenza immunisation during pregnancy in Nepal: a phase 4, randomised, placebo-controlled trial. *Lancet Infect Dis* **2017**; 17:981–9.
- Steinhoff MC, Omer SB, Roy E, et al. Influenza immunization in pregnancy—antibody responses in mothers and infants. *N Engl J Med* **2010**; 362:1644–6.
- Nunes MC, Cutland CL, Jones S, et al. Duration of infant protection against influenza illness conferred by maternal immunization: secondary analysis of a randomized clinical trial. *JAMA Pediatr* **2016**; 170:840–7.
- Nunes MC, Cutland CL, Dighe B, et al. Kinetics of hemagglutination-inhibiting antibodies following maternal influenza vaccination among mothers with and those without HIV infection and their infants. *J Infect Dis* **2015**; 212: 1976–87.
- Nunes MC, Weinberg A, Cutland CL, et al. Neutralization and hemagglutination-inhibition antibodies following influenza vaccination of HIV-infected and HIV-uninfected pregnant women. *PLoS One* **2018**; 13: e0210124.
- Dhar N, Kwatra G, Nunes MC, et al. Hemagglutinin-stalk antibody responses following trivalent inactivated influenza vaccine immunization of pregnant women and association with protection from influenza virus illness. *Clin Infect Dis* **2020**; 71:1072–9.
- Benowitz I, Esposito DB, Gracey KD, Shapiro ED, Vázquez M. Influenza vaccine given to pregnant women reduces hospitalization due to influenza in their infants. *Clin Infect Dis* **2010**; 51:1355–61.
- Dabrera G, Zhao H, Andrews N, et al. Effectiveness of seasonal influenza vaccination during pregnancy in preventing influenza infection in infants, England, 2013/14. *Euro Surveill* **2014**; 19:20959.
- Poehling KA, Szilagyi PG, Staat MA, et al. Impact of maternal immunization on influenza hospitalizations in infants. *Am J Obstet Gynecol* **2011**; 204(6 Suppl 1): S141–8.

13. Shakib JH, Korgenski K, Presson AP, et al. Influenza in infants born to women vaccinated during pregnancy. *Pediatrics* **2016**; 137:e20152360.
14. Molgaard-Nielsen D, Fischer TK, Krause TG, Hviid A. Effectiveness of maternal immunization with trivalent inactivated influenza vaccine in pregnant women and their infants. *J Intern Med* **2019**; 286:469–80.
15. Walker JL, Zhao H, Dabrera G, et al. Assessment of effectiveness of seasonal influenza vaccination during pregnancy in preventing influenza infection in infants in England, 2013–2014 and 2014–2015. *J Infect Dis* **2020**; 221:16–20.
16. Thompson MG, Kwong JC, Regan AK, et al. Influenza vaccine effectiveness in preventing influenza-associated hospitalizations during pregnancy: a multi-country retrospective test negative design study, 2010–2016. *Clin Infect Dis* **2019**; 68:1444–53.
17. Bishop K, McMorrow M, Meiring S, et al. An evaluation of an influenza vaccination campaign targeting pregnant women in 27 clinics in two provinces of South Africa, 2015–2018. *BMC Health Serv Res* **2021**; 21:941.
18. Jernigan DB, Lindstrom SL, Johnson JR, et al. Detecting 2009 pandemic influenza A (H1N1) virus infection: availability of diagnostic testing led to rapid pandemic response. *Clin Infect Dis* **2011**; 52(Suppl 1):S36–43.
19. McAnerney JM, Walaza S, Tempia S, et al. Estimating vaccine effectiveness in preventing laboratory-confirmed influenza in outpatient settings in South Africa, 2015. *Influenza Other Respir Viruses* **2017**; 11:177–81.
20. The Crick Worldwide Influenza Centre, UK. [https://www.crick.ac.uk/sites/default/files/2018-07/september\\_2016\\_interim\\_report.pdf](https://www.crick.ac.uk/sites/default/files/2018-07/september_2016_interim_report.pdf). Accessed 27 March 2022.
21. The Crick Worldwide Influenza Centre, UK. [https://www.crick.ac.uk/sites/default/files/2018-10/September%202018%20interim%20report\\_opt.pdf](https://www.crick.ac.uk/sites/default/files/2018-10/September%202018%20interim%20report_opt.pdf). Accessed 27 March 2022.
22. The Crick Worldwide Influenza Centre, UK. [https://www.crick.ac.uk/sites/default/files/2018-07/crick\\_sh2017\\_vcm\\_report\\_to\\_post.pdf](https://www.crick.ac.uk/sites/default/files/2018-07/crick_sh2017_vcm_report_to_post.pdf). Accessed 27 March 2022.
23. The Crick Worldwide Influenza Centre, UK. [https://www.crick.ac.uk/sites/default/files/2018-07/crick\\_sep2015\\_vcm\\_report\\_to\\_post.pdf](https://www.crick.ac.uk/sites/default/files/2018-07/crick_sep2015_vcm_report_to_post.pdf). Accessed 27 March 2022.
24. National Institute for Communicable Diseases, South Africa. About us. <https://www.nicd.ac.za/about-us/>. Accessed 27 March 2022.
25. Madhi SA, Maskew M, Koen A, et al. Trivalent inactivated influenza vaccine in African adults infected with human immunodeficient virus: double blind, randomized clinical trial of efficacy, immunogenicity, and safety. *Clin Infect Dis* **2011**; 52:128–37.
26. McLean HQ, Thompson MG, Sundaram ME, et al. Influenza vaccine effectiveness in the United States during 2012–2013: variable protection by age and virus type. *J Infect Dis* **2015**; 211:1529–40.
27. Skowronski DM, Janjua NZ, Sabaiduc S, et al. Influenza A/subtype and B/lineage effectiveness estimates for the 2011–2012 trivalent vaccine: cross-season and cross-lineage protection with unchanged vaccine. *J Infect Dis* **2014**; 210:126–37.
28. <http://www.statssa.gov.za/?p=13100>.

# Immunogenicity of tetanus, diphtheria and acellular pertussis vaccination among pregnant women living with and without HIV

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**Objective:** Vaccination during pregnancy with tetanus–diphtheria–acellular pertussis (Tdap) vaccine is recommended to protect the young infants against pertussis. There is a paucity of data on immune responses to Tdap in pregnant women with HIV (PWWH), and its impact on the protection of their infants has not been described.

**Methods:** In an open label phase IV clinical trial in South Africa, we evaluated the immunogenicity and safety of Tdap in PWWH compared with HIV-uninfected women. Antigen-specific immunoglobulin G (IgG) to pertussis toxoid, filamentous haemagglutinin, pertactin, fimbriae, diphtheria and tetanus were measured by electrochemiluminescence-based multiplex assay.

**Results:** Overall, 91 PWWH and 136 HIV-uninfected pregnant women were enrolled. All PWWH were on antiretroviral treatment and 94.5% had HIV viral loads <40 copies per millilitre. Antibody levels prevaccination were lower among PWWH compared with HIV-uninfected women for all antigens. At 1 month postvaccination PWWH compared with HIV-uninfected women had lower fold-increase and antibody concentrations for all epitopes. Also, a lower proportion of PWWH achieved  $\geq 4$ -fold increase from pre to postvaccination for pertussis toxoid and pertactin, or diphtheria IgG levels  $\geq 0.1$  IU/ml and  $\geq 1$  IU/ml postvaccination. Adverse events postvaccination were similar in PWWH and HIV-uninfected.

**Conclusion:** Tdap vaccination was safe and immunogenic. PWWH had, however, attenuated humoral immune responses, which could affect the effectiveness of protecting their infants against pertussis compared with those born to women without HIV.

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## Introduction

Pertussis (whooping cough) is a highly contagious vaccine-preventable respiratory tract disease, caused by the bacterium *Bordetella pertussis*. Pertussis can affect people of all ages; however, young unimmunised or partially immunised infants are the most vulnerable group for severe illness and death [1]. In 2014, there were an estimated 24.1 million pertussis cases and 160 700 associated deaths worldwide, with the African region having the largest burden (7.8 million cases and 92 500 deaths). Globally, infants (<12 months of age) account for 5.1 million cases and approximately 85,900 deaths annually [1].

To reduce the susceptibility window period for pertussis between birth and the third dose of the primary vaccination series in infancy, acellular pertussis (aP)-containing vaccines are recommended during pregnancy. Following the initial recommendations in 2011–2012 from the UK and the USA, many other countries have implemented routine vaccination with aP-containing vaccine during pregnancy [2]. Nevertheless, use of aP-containing vaccines during pregnancy has not as yet been adopted into public immunization programs of any African country. Studies from countries with low prevalence of human immunodeficiency virus (HIV) infection in pregnant women report that aP vaccination induces good immune responses during pregnancy and the active transplacental transfer of pertussis immunoglobulin G (IgG) to the foetus. Furthermore, vaccination with aP-containing vaccines is safe to the women, with no adverse pregnancy outcomes of concern [3]. Vaccine effectiveness estimates of maternal vaccination against severe pertussis in their young infants (<3 months of age) is 91% to 94% for pertussis-associated hospitalization and 95% for pertussis-related deaths [4].

Pregnant women, and adults in general, living with HIV have lower humoral immune responses to vaccination, compared with their HIV-uninfected counterparts [5,6,7]. Consequently, it is necessary to investigate the immune responses of aP-containing vaccines in pregnant women with HIV (PWWH), to understand the relative protection of their infants against pertussis compared with offspring born to women without HIV. This is pertinent in many sub-Saharan African countries where there remains a high prevalence of HIV in women of childbearing age. South Africa has an estimated 8.4 million people living with HIV and in 2019 the national antenatal HIV prevalence was estimated at 30% [8].

We undertook an open label phase IV clinical trial to evaluate the immunogenicity and safety of an adult formulation of tetanus–diphtheria–acellular pertussis (Tdap) vaccine in PWWH compared with HIV-uninfected women.

## Methods

Pregnant women attending antenatal care at clinics in Soweto, South Africa, were screened for enrolment into the study. Healthy, pregnant women 18–39 years of age, 20–36 weeks of gestational age of a singleton pregnancy, considered to be at low risk for complications, and documented to be HIV-infected or HIV-uninfected were eligible for enrolment (full inclusion and exclusion criteria in supplement, <http://links.lww.com/QAD/C991>). In South Africa, pregnant women attending public antenatal clinics are offered tetanus-toxoid containing vaccines and seasonal influenza vaccines, but not Tdap [9]. After written informed consent was obtained, and detailed demographic, obstetric and medical information was collected, women received commercially available Adacel (Sanofi, France) vaccine.

Venous blood was collected just prior to vaccination and at 28–35 days postvaccination. Solicited injection-site and systemic adverse events (AEs) were recorded on a diary card for seven days postvaccination. Unsolicited AEs and serious AEs (SAEs) were collected for the duration of the study. Serum was tested by electrochemiluminescence-based multiplex assay (using technology from Meso Scale Diagnostics) for IgG against pertussis-toxoid (PT), filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae (FIM) (reported as ELISA units [EU]/ml), diphtheria and tetanus (reported as international units [IU]/ml). The method was developed and validated at Sanofi and has been described in detail [10]. All assays were performed in the laboratories of Sanofi in Swiftwater, Pennsylvania, USA, in a blinded manner to the HIV status of the participant.

The study was designed with two co-primary objectives: describe antibody responses to all Tdap epitopes in PWWH compared with HIV-uninfected pregnant women before and 1 month after Adacel vaccination; describe IgG responses to all Tdap and Hexavalent antigens in infants born to mothers who received Adacel and those born to mothers not vaccinated, stratified by maternal HIV status. A sample size of 90 vaccinated PWWH and their infants, 90 infants born to unvaccinated PWWH, 135 HIV-uninfected vaccinated women and their infants, and 135 infants born to HIV-uninfected unvaccinated women would provide 80% power to detect the difference in IgG levels both in the women and the infants (details in supplement, <http://links.lww.com/QAD/C991>). The current analysis describes the immunogenicity results of Tdap in the women, and safety analyses including outcomes reported from vaccination to postvaccination visit.

Participants' characteristics were compared between PWWH and HIV-uninfected women. Geometric mean antibody concentrations (GMCs) and the corresponding 95% confidence interval (CI) were estimated using logarithmic transformation, the percentage of participants

achieving  $\geq 4$ -fold increase in PT, FHA, PRN and FIM IgG levels from pre to postvaccination, besides the percentage of those with tetanus and diphtheria IgG  $\geq 0.1$  IU/ml and  $\geq 1$  IU/ml was calculated, and compared between PWWH and HIV-uninfected women by univariate and multivariate regression analyses. Postvaccination immunogenicity analyses including only women in whom the postvaccination visits were done within a 28–35 days window period were performed. Reporting on AEs and SAEs were descriptive.

The study was approved by the University of the Witwatersrand Human Research Ethics Committee (210706). ClinicalTrials.gov identifier: NCT05264662.

### Results

From 7 March to 12 September 2022, 227 pregnant women were enrolled and provided prevaccination serum, including 91 PWWH and 136 HIV-uninfected (study consort diagram in supplement, Figure S1, <http://links.lww.com/QAD/C986>). The participants' characteristics were similar between the two study groups, except for PWWH being older (30.7 years) and less likely to be primigravida (5.5%) compared with HIV-uninfected women (27.3 years and 27.9%,  $P < 0.001$  for both comparisons), Table 1. All PWWH were on antiretroviral treatment (ART), the median CD4+ T-lymphocyte

count was 561.5cells/ $\mu$ L and 94.5% had HIV viral loads  $< 40$  copies/ml at the time of vaccination. Overall, 220 women had a postvaccination visit, including 210 (95.5%) within 28–35 days postvaccination.

Prevaccination pertussis specific epitope IgG levels were lower among PWWH compared with HIV-uninfected women for all four pertussis antigens, Table 2. Despite increase in IgG levels in both groups 1 month postvaccination, PWWH had significantly lower fold-rise and antibody levels compared with HIV-uninfected women. The proportion of women achieving  $\geq 4$ -fold increase in IgG from pre to postvaccination was also lower in PWWH compared with women without HIV for PT and PRN, Table 2. PWWH achieving  $\geq 4$ -fold increase in PRN IgG had higher median CD4+ T-lymphocyte count (585.5cells/ $\mu$ L IQR: 434, 808.5) compared with those not reaching that threshold (373.5 cells/ $\mu$ L IQR: 277, 633;  $P = 0.034$ ), although no such effect was observed for the other antigens.

For diphtheria and tetanus, PWWH had lower pre and postvaccination antibody levels. With  $> 85\%$  of all women having already prevaccination antibody levels for tetanus  $\geq 1$  IU/ml. While all women achieved IgG levels  $\geq 0.1$  IU/ml and  $\geq 1$  IU/ml postvaccination for tetanus, a lower proportion of PWWH than HIV-uninfected women achieved those thresholds for diphtheria, Table S1 in supplement, <http://links.lww.com/QAD/C987>.

**Table 1. Characteristics at enrolment for all vaccinated women by HIV status.**

|  | Overall N=227 | HIV-N=136  | PWWH N=91        | P-value   |
|--|---------------|------------|------------------|-----------|
| Number of Black African race (%)   | 227 (100)     | 136 (100)  | 91 (100)         | n.a.      |
| Mean age (standard deviation); years   | 28.7 (5.5)    | 27.3 (5.5) | 30.7 (4.9)       | $< 0.001$ |
| Mean MUAC (standard deviation); cm   | 29.4 (3.9)    | 29.4 (3.9) | 29.3 (4.0)       | 0.88      |
|  | [195]         | [112]      | [83]             |           |
| Mean BMI (standard deviation)  | 30.8 (6.1)    | 30.8 (5.7) | 30.7 (6.5)       | 0.98      |
|  | [194]         | [111]      | [83]             |           |
| Mean gestational age (standard deviation); weeks                             | 28.7 (4.1)    | 28.9 (3.9) | 28.2 (4.4)       | 0.29      |
| Number of primigravida (%)   | 43 (18.9)     | 38 (27.7)  | 5 (5.5)          | $< 0.001$ |
| Median gravidity (IQR)   | 2 (1.5, 2.5)  | 2 (1, 3)   | 3 (2.5, 3.5)     | $< 0.001$ |
| Number smoking during pregnancy (%)  | 10 (4.4)      | 4 (2.9)    | 6 (6.6)          | 0.20      |
| Number drinking alcohol during pregnancy (%)                                 | 7 (3.1)       | 3 (2.2)    | 4 (4.4)          | 0.44      |
| Number with less than 12 years of education (%)                              | 65 (28.6)     | 32 (23.4)  | 33 (36.3)        | 0.11      |
| Number with 12 years of education (%)  | 135 (59.5)    | 86 (62.8)  | 49 (53.9)        |           |
| Number with tertiary education (%)   | 28 (12.3)     | 19 (13.9)  | 9 (9.9)          |           |
| Number vaccinated with at least one TT dose during this pregnancy (%)        | 117 (51.5)    | 65 (47.8)  | 52 (57.1)        | 0.17      |
| Median TT doses during this pregnancy (IQR)                                  | 1 (0, 1)      | 0 (0, 1)   | 1 (0, 1)         | 0.09      |
| Number on antiretroviral treatment (%) <sup>a</sup>                          | n.a.          | n.a.       | 91 (100)         | n.a.      |
| HIV-1 viral load at enrolment $< 40$ copies per milliliter (%)               | n.a.          | n.a.       | 86 (94.5)        | n.a.      |
| Median CD4+ T-lymphocyte count (IQR); cells per microliter                   | n.a.          | n.a.       | 561.5 (383, 788) | n.a.      |
| Mean days between vaccination and postvaccination visit (Standard deviation) | 28.9 (2.8)    | 29.0 (2.7) | 28.7 (3.0)       | 0.45      |
|  | [220]         | [132]      | [88]             |           |
| Number of postvaccination visits within window period (%) <sup>b</sup>       | 210 (95.5)    | 125 (94.7) | 85 (96.6)        | 0.74      |
|  | [220]         | [132]      | [88]             |           |
| Number of deliveries before postvaccination visit                            | 9             | 5          | 4                | n.a.      |

BMI, body mass index; HIV, HIV-uninfected pregnant women; IQR, interquartile range; MUAC, mid-upper arm circumference; n.a., nonapplicable; PWWH, pregnant women with HIV; TT, tetanus toxoid containing vaccine. Number in brackets represent the number of participants with available information if different from N and information in parentheses was calculated taking this in consideration.

<sup>a</sup>89 were on first line therapy (19 on Tenofovir, Emtricitabine, Efavirenz fixed dose combination, and 70 on Tenofovir, Lamivudine, Dolutegravir [TLD] fixed dose combination), 1 was on second line therapy (Lamivudine/Zidovudine/Atazanavir/Ritonavir) and 1 on third line therapy (Darunavir/Ritonavir/TLD).

<sup>b</sup>Per-protocol visits window period, 28–35 days between visits.

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**Table 2. Immune responses among pregnant women living with and without HIV to pertussis antigens.**

|   | PERTUSSIS PT                    |                              |                      | PERTUSSIS FHA                    |                                 |                      | PERTUSSIS FIM                     |                                 |                      | PERTUSSIS PRN                    |                              |                      |
|---|---------------------------------|------------------------------|----------------------|----------------------------------|---------------------------------|----------------------|-----------------------------------|---------------------------------|----------------------|----------------------------------|------------------------------|----------------------|
|   | HIV-                            | PWWH                         | P-value/<br>aP-value | HIV-                             | PWWH                            | P-value/<br>aP-value | HIV-                              | PWWH                            | P-value/<br>aP-value | HIV-                             | PWWH                         | P-value/<br>aP-value |
| GMCs pre-vaccination (95% CI)                                     | 10.3<br>(8.6, 12.4)<br>[134]    | 7.2<br>(5.9, 9.0)<br>[89]    | 0.015/0.033          | 26.9<br>(23.5, 30.7)<br>[136]    | 18.9<br>(15.1, 23.6)<br>[91]    | 0.005/0.010          | 10.9<br>(8.6, 13.7)<br>[136]      | 4.6<br>(3.4, 6.2)<br>[91]       | <0.001/0.021         | 4.9<br>(3.9, 6.1)<br>[136]       | 3.2<br>(2.5, 4.1)<br>[91]    | 0.012/0.027          |
| GMCs post-vaccination (95% CI)                                    | 98.7<br>(87.0, 111.9)<br>[132]  | 50.8<br>(41.1, 62.6)<br>[88] | <0.001/<0.001        | 486.3<br>(423.9, 562.4)<br>[132] | 241.2<br>(190.7, 305.0)<br>[88] | <0.001/<0.001        | 799.5<br>(597.1, 1070.6)<br>[132] | 214.7<br>(146.1, 315.5)<br>[88] | <0.001/<0.001        | 170.8<br>(134.3, 217.2)<br>[132] | 66.0<br>(45.1, 96.5)<br>[88] | <0.001/<0.001        |
| GMCs at 28–35 days post-vaccination (95% CI) <sup>a</sup>         | 101.7<br>(89.4, 115.6)<br>[125] | 50.9<br>(41.1, 63.1)<br>[85] | <0.001/<0.001        | 483.0<br>(419.1, 556.6)<br>[125] | 236.8<br>(185.9, 301.7)<br>[85] | <0.001/<0.001        | 822.2<br>(608.6, 1110.8)<br>[125] | 231.6<br>(158.8, 337.9)<br>[85] | <0.001/0.001         | 178.4<br>(140.1, 227.3)<br>[125] | 66.6<br>(45.7, 96.9)<br>[85] | <0.001/<0.001        |
| Geometric mean fold rise (95% CI)                                 | 9.8<br>(8.4, 11.5)<br>[130]     | 7.1<br>(5.8, 8.6)<br>[86]    | 0.010/0.011          | 18.1<br>(15.2, 21.5)<br>[132]    | 12.7<br>(10.0, 16.2)<br>[88]    | 0.019/0.006          | 74.8<br>(59.4, 94.4)<br>[132]     | 47.2<br>(34.9, 63.9)<br>[88]    | 0.016/0.037          | 34.6<br>(28.0, 42.8)<br>[132]    | 20.4<br>(14.6, 28.6)<br>[88] | 0.006/0.005          |
| Geometric mean fold rise (95% CI) <sup>a</sup>                    | 9.8<br>(8.3, 11.5)<br>[124]     | 7.1<br>(5.8, 8.7)<br>[83]    | 0.017/0.017          | 17.7<br>(14.8, 21.2)<br>[125]    | 12.3<br>(9.6, 15.8)<br>[85]     | 0.018/0.008          | 74.3<br>(58.3, 94.7)<br>[125]     | 49.9<br>(37.1, 67.2)<br>[85]    | 0.040/0.073          | 34.6<br>(27.8, 43.2)<br>[125]    | 21.0<br>(15.0, 29.4)<br>[85] | 0.011/0.011          |
| ≥4-fold increase from pre to post-vaccination; n (%)              | 117 (90.0)<br>[130]             | 65 (75.6)<br>[86]            | 0.004/0.003          | 122 (92.4)<br>[132]              | 77 (87.5)<br>[88]               | 0.23/0.11            | 130 (98.5)<br>[132]               | 83 (94.3)<br>[88]               | 0.11/0.15            | 129 (97.7)<br>[132]              | 76 (86.4)<br>[88]            | 0.004/0.012          |
| ≥4-fold increase from pre to post-vaccination; n (%) <sup>a</sup> | 111 (89.5)<br>[124]             | 62 (74.7)<br>[83]            | 0.006/0.003          | 115 (92.0)<br>[125]              | 74 (86.9)<br>[85]               | 0.25/0.13            | 123 (98.4)<br>[125]               | 81 (95.3)<br>[85]               | 0.21/0.26            | 122 (97.6)<br>[125]              | 74 (87.1)<br>[85]            | 0.007/0.021          |

95% CI, 95% confidence interval; FHA, filamentous haemagglutinin; FIM, fimbriae; GMCs, geometric means concentrations; HIV, HIV-uninfected pregnant women; PRN, pertactin; PT, pertussis toxin; PWWH, pregnant women with HIV. aP-value: P-value adjusted for age and primigravida (yes/no). Number in brackets represent the number of participants with available information and information in parentheses was calculated taking this in consideration.

<sup>a</sup>Only women who attended the visits within the protocol defined window period were included.

During the first week postvaccination, the most commonly reported local injection site reaction was itching by both PWWH (51.1%) and HIV-uninfected women (43.2%); weakness/tiredness was the most frequent systemic reaction reported by those living with (37.8%) and without (33.3%) HIV, Table S2 in supplement, <http://links.lww.com/QAD/C988>. There were no differences in the frequency of solicited AEs between the two study groups. Overall, 34 SAEs were reported, including seven episodes of gestational hypertension and four premature labour events, Table S3 in supplement, <http://links.lww.com/QAD/C989>. However, of all the SAEs only one premature labour that occurred two days after vaccination of a HIV-uninfected woman was considered as being possibly related to vaccination based solely on temporal association. AEs reported from vaccination to 1 month postvaccination are shown in Table S4 in supplement, <http://links.lww.com/QAD/C990>.

## Discussion

In this trial, PWWH on ART and with well controlled HIV infection had lower antibody levels for all assessed antigens compared with HIV-uninfected pregnant women at enrolment. Tdap elicited an antibody response in both study groups, although PWWH continued to display lower antibody levels and lower fold-rise postvaccination. As no correlate of protection is available for pertussis, the surrogate for measuring immune response was defined as  $\geq 4$ -fold increase in antibody levels from pre to postvaccination [11]; using this criterion  $>75\%$  of PWWH seroconverted to PT. Despite a less robust immune response among PWWH, vaccination might still confer some protection to in-utero HIV exposed young infants, through transplacentally transferred antibodies. Our group had previously shown that HIV exposed infants (born to unvaccinated mothers) had similar PT and FHA antibody levels compared with HIV-unexposed infants prior to their first dose of pertussis-containing vaccine at seven weeks of age, suggesting that unvaccinated mothers had similar transplacental antibody transfer independently of maternal HIV infection status [11].

A systematic review and meta-analysis of data from 37 low- and middle-income countries, described that the case fatality rate among confirmed pertussis cases was 7.2% during the first year of life in the studies reporting deaths, and that HIV exposure was associated with higher pertussis incidence, higher rates of hospitalisation and pertussis-related deaths (although data were limited by the number of countries reporting HIV status) [12]. Due to the higher burden of pertussis in infants born to mothers living with HIV, vaccinating PWWH even if with lower immune response may impact disease burden. We will

evaluate the antibody concentration in the infants born to the mothers in our study and comparing those to infants born to unvaccinated mothers will allow us to better predict protection against infant disease. A recent hospital-based pneumonia surveillance study in South Africa identified adults living with HIV as an important group at risk of severe pertussis, and alerted to the need for more regular booster vaccination in this population, including pregnant women [13].

Some studies reported that low HIV viral loads, and high CD4<sup>+</sup> T-lymphocyte counts, correlated with improved immune responses to influenza vaccines in adults living with HIV [14,15]. In our study, only for PRN IgG PWWH achieving  $\geq 4$ -fold increase had higher median CD4<sup>+</sup> T-lymphocyte counts than those not achieving. Additionally, no effect on immune response was observed according to viral load (data not shown); however, 94.5% of the PWWH had undetectable viral load.

A limitation of our study was the noninclusion of a placebo control group, although since the primary objective of the current analysis was to compare immune responses of PWWH to those of HIV-uninfected, the control group would be more relevant for a complete safety assessment. Our study, however, supports the tolerability of Tdap during pregnancy and no increased reactogenicity was detected in PWWH. Randomized controlled trials and large observational studies have not identified any SAEs or adverse pregnancy outcomes associated with Tdap during pregnancy [4]. Also, we did not collect information on prior pertussis vaccination. A combined infant whole-cell pertussis vaccine was introduced in South Africa in 1957 and in 2009 the country changed from whole-cell pertussis vaccines to acellular pertussis-containing vaccine given at 6, 10, and 14 weeks of age followed by a booster at 18 months of age. Since adolescent and adult boosters are not routinely offered in South Africa we expect that both study groups would have similar vaccination histories.

Vaccination of PWWH may benefit from different approaches, such as more immunogenic vaccines, high dose vaccines or more than one dose possibly with different schedules, to increase the level of antibodies transfer to the foetus and confer protection to the HIV exposed infant. We will be reporting on the infants' follow-up regarding antibody levels pre and postprimary vaccination series and booster vaccination.

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### Conflicts of interest

M.C.N. reports grants from the Bill & Melinda Gates Foundation, European & Developing Countries Clinical Trials Partnership, Pfizer, AstraZeneca, and Sanofi; and personal fees from Pfizer and Sanofi. S.A.M. reports grants and personal fees from the Bill & Melinda Gates Foundation, and grants from the South African Medical Research Council, Novavax, Pfizer, Minervax, and European & Developing Countries Clinical Trials Partnership. The other authors report no conflicts of interest.

### References

1. Yeung KHT, Duclos P, Nelson EAS, Hutubessy RCW. **An update of the global burden of pertussis in children younger than 5 years: a modelling study.** *Lancet Infect Dis* 2017; **17**:974–980.
2. Abu-Raya B, Forsyth K, Halperin SA, Maertens K, Jones CE, Heining U, et al. **Vaccination in pregnancy against pertussis: a consensus statement on behalf of the global pertussis initiative.** *Vaccines (Basel)* 2022; **10**:1990–2006.
3. Furuta M, Sin J, Ng ESW, Wang K. **Efficacy and safety of pertussis vaccination for pregnant women - a systematic review of randomised controlled trials and observational studies.** *BMC Pregnancy Childbirth* 2017; **17**:390.
4. Vygen-Bonnet S, Hellenbrand W, Garbe E, Von Kries R, Bogdan C, Heining U, et al. **Safety and effectiveness of acellular pertussis vaccination during pregnancy: a systematic review.** *BMC Infect Dis* 2020; **20**:136–158.
5. Nunes MC, Cutland CL, Dighero B, Bate J, Jones S, Hugo A, et al. **Kinetics of hemagglutination-inhibiting antibodies following maternal influenza vaccination among mothers with and those without HIV infection and their infants.** *J Infect Dis* 2015; **212**:1976–1987.
6. Crum-Cianflone NF, Wallace MR. **Vaccination in HIV-infected adults.** *AIDS Patient Care STDS* 2014; **28**:397–410.
7. Madhi SA, Moodley D, Hanley S, Archary M, Hoosain Z, Lalloo U, et al. **Immunogenicity and safety of a SARS-CoV-2 recombinant spike protein nanoparticle vaccine in people living with and without HIV-1 infection: a randomised, controlled, phase 2A/2B trial.** *Lancet HIV* 2022; **9**:309–322.
8. Woldeesenbet SA, Lombard C, Manda S, Kufa T, Ayalew K, Cheyip M, et al. **The 2019 National Antenatal Sentinel HIV Survey, South Africa, National Department of Health.** Available at: [https://www.nicd.ac.za/wp-content/uploads/2021/11/Antenatal-survey-2019-report\\_FINAL\\_27April21.pdf](https://www.nicd.ac.za/wp-content/uploads/2021/11/Antenatal-survey-2019-report_FINAL_27April21.pdf).
9. **Standard Treatment Guidelines and Essential Medicines List for South Africa, 2020.** Available at: <https://www.knowledgethub.org.za/e-library>.
10. Varghese K, Bartlett W, Zheng L, Bookhout S, Vincent D, Huleatt J, et al. **A new electrochemiluminescence-based multiplex assay for the assessment of human antibody responses to *Bordetella pertussis* vaccines.** *Infect Dis Ther* 2021; **10**:2539–2561.
11. Simani OE, Izu A, Violari A, Cotton MF, Van Niekerk N, Adrian PV, et al. **Effect of HIV-1 exposure and antiretroviral treatment strategies in HIV-infected children on immunogenicity of vaccines during infancy.** *AIDS* 2014; **28**:531–541.
12. Muloowa R, Kagina BM, Engel ME, Hussey GD. **The burden of laboratory-confirmed pertussis in low-and middle-income countries since the inception of the Expanded Programme on Immunisation (EPI) in 1974: a systematic review and meta-analysis.** *BMC Med* 2020; **18**:233.
13. Wolter N, Cohen C, Tempia S, Walaza S, Moosa F, Du Plessis M, et al. **Epidemiology of pertussis in individuals of all ages hospitalized with respiratory illness in South Africa, January 2013–December 2018.** *Clin Infect Dis* 2021; **73**:E745–E753.
14. Yamanaka H, Teruya K, Tanaka M, Kikuchi Y, Takahashi T, Kimura S, et al. **Efficacy and immunologic responses to influenza vaccine in HIV-1-infected patients.** *J Acquir Immune Defic Syndr* 2005; **39**:167–173.
15. Evison J, Farese S, Seitz M, Uehlinger DE, Furrer H, Mühlemann K. **Randomized, double-blind comparative trial of subunit and virosomal influenza vaccines for immunocompromised patients.** *Clin Infect Dis* 2009; **48**:1402–1412.



## COVID-19

With the advent of the COVID-19 pandemic a significant part of my research from the beginning of 2020 to the end of my stay at Wits Vida, in July 2022, was devoted to establish how the pandemic impacted other respiratory pathogens, the immune response to SARS-CoV-2 infections and the burden of COVID-19 in specific populations such as pregnant women, people living with HIV and health care workers (HCW). We were able to very quickly adapt our hospital surveillance for respiratory diseases to include SARS-CoV-2 and expand the enrolled population to adult patients and pregnant women. The laboratory of Wits Vida performed SARS-CoV-2 PCR testing from the very beginning of the pandemic and until May 2020 was the only laboratory at the hospital performing routine testing. Besides respiratory samples we also collected many blood samples that were crucial for internal projects and important international collaborations (Dejnirattisai et al. 2022; C. Liu et al. 2021).

I undertook a study on targeted post-mortem Minimal Invasive Tissue Sampling (MITS) among respiratory illness-related deaths. Fatal adult cases suspected of COVID-19 were investigated by MITS of the lung, heart, and liver. We included 75 COVID-19-related deaths (COVID+) and 42 non-COVID-19-related deaths (COVID-) and found that the prevalence of HIV-infection was lower in COVID+ (27%) than in the COVID- (64%), mycobacterium tuberculosis detection was also less common among COVID+ (3% vs 13%). Lung histopathology findings showed differences between COVID+ and COVID- in the severity of the morphological appearance of Type-II pneumocytes, alveolar injury and repair initiated by SARS-CoV-2 infection. In the liver necrotising granulomatous inflammation was more common among COVID+. No differences were found in heart analyses. The prevalence of bacterial co-infections was higher in COVID+. Most indicators of respiratory distress syndrome were undifferentiated between COVID+ and COVID- except for Type-II pneumocytes (Marta C. Nunes, Hale, et al. 2022).

Among pregnant women I investigated SARS-CoV-2 infection antenatally and in women presenting for delivery. We found that antenatal SARS-CoV-2 infection, mostly detected in the third trimester of pregnancy, was associated with 1.6-higher odds of having a low-birthweight newborn. A similar observation was evident when restricting the analysis to symptomatic infections, i.e. COVID-19 cases. Although most of the participants in our study had mild disease, 21 of the SARS-CoV-2 infected women were hospitalized – among whom one died due to COVID-19 pneumonia (Marta C Nunes, Jones, et al. 2022). We also demonstrated that intrapartum, SARS-CoV-2 infection was not associated with fetal death, preterm birth, low birth weight, or pregnancy-induced complications (Marta C. Nunes, Jones, et al. 2022).

An important cohort that I established during the COVID-19 pandemic was among HCW working at Chris Hani Baragwanath Hospital. I designed a longitudinal intense follow-up strategy to describe the full spectrum of disease among HCW, including the extent of asymptomatic infections. During the first COVID-19 wave, we identified 137 (34.6%) PCR-confirmed SARS-CoV-2 infections among the 396 HCWs enrolled in the study. This increased to 41.9% when including cases manifesting sero-positivity at the time of enrolment or sero-response during the study. We also identified that among the SARS-CoV-2 PCR-confirmed cases, 10.4% HCWs remained asymptomatic, 30.4% were pre-symptomatic and 59.3% were symptomatic. Symptomatic cases compared with asymptomatic had lower PCR cycle threshold values at diagnosis and longer duration of PCR-positivity (Marta C Nunes et al. 2021).

With the first detection of the Omicron variant in South Africa we investigated symptomatic infections due to this variant among the HCW participating in our cohort study. Due to the high vaccination coverage and previous SARS-CoV-2 infection rate we were able to assess the protection afforded by vaccination or prior infection. Our study designed also allowed us to measure full-spike protein IgG antibodies on samples collected from 62% of the participants prior or at the time of the symptomatic illness visit and we found that HCW in whom Omicron was not detected had significant higher anti-spike IgG levels compared with those infected by Omicron suggesting a protective effect of these antibodies. Furthermore, to investigate which combinations of covariates significantly modulate Omicron infection, we performed a recursive partitioning approach in the form of conditional inference trees and found that the lowest probability of having an Omicron infection during the study period was among those previously infected or those without prior confirmed infection but with high antibody levels, presumably induced by vaccination. A threshold of 1549 anti-spike IgG binding units emerged as suggestive of significant reduction in the risk of symptomatic Omicron infection (Marta C. Nunes, Mbotwe-Sibanda, et al. 2022). We also investigated the symptoms of post-acute sequelae of SARS-CoV-2 infection among the infected HCW (Mbotwe-Sibanda et al. 2024).

Attached two papers, one describing the impact of antenatal SARS-CoV-2 infection on pregnancy outcomes and the other on the high force of infection among HCW during the first pandemic wave.

# Antepartum SARS-CoV-2 infection and adverse birth outcomes in South African women

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**Background** SARS-CoV-2 infection in pregnant women has been associated with severe illness in the women and higher rates of premature delivery. There is, however, paucity of data on the impact of the timing of SARS-CoV-2 infection and on symptomatic or asymptomatic infections on birth outcomes. Data from low-middle income settings is also lacking.

**Methods** We conducted a longitudinal study from April 2020 to March 2021, in South Africa, where symptomatic or asymptomatic pregnant women were investigated for SARS-CoV-2 infection during the antepartum period. We aimed to evaluate if there was an association between antepartum SARS-CoV-2 infection on birth outcomes. SARS-CoV-2 infection was investigated by nucleic acid amplification test (NAAT), histological examination was performed in a sub-set of placentas.

**Results** Overall, 793 women were tested for SARS-CoV-2 antenatally, including 275 (35%) who were symptomatic. SARS-CoV-2 infection was identified in 138 (17%) women, of whom 119 had symptoms (COVID-19 group) and 19 were asymptomatic. The 493 women who were asymptomatic and had a negative SARS-CoV-2 NAAT were used as the referent comparator group for outcomes evaluation. Women with COVID-19 compared with the referent group were 1.66-times (95% confidence interval (CI)=1.02-2.71) more likely to have a low-birthweight newborn (30% vs 21%) and 3.25-times more likely to deliver a very low-birthweight newborn (5% vs 2%). Similar results for low-birthweight were obtained comparing women with SARS-CoV-2 confirmed infection (30%) with those who had a negative NAAT result (22%) independent of symptoms presentation. The placentas from women with antenatal

SARS-CoV-2 infection had higher percentage of chorangiomas (odds ratio (OR)=3.40, 95% CI=1.18-.84), while maternal vascular malperfusion was more frequently identified in women who tested negative for SARS-CoV-2 (aOR=0.28, 95% CI=0.09-0.89).

**Conclusions** Our study demonstrates that in a setting with high HIV infection prevalence and other comorbidities antenatal SARS-CoV-2 infection was associated with low-birthweight delivery.

The current coronavirus disease 2019 (COVID-19) pandemic, caused by the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), was declared a global health emergency in March 2020. By June 30, 2022, 12.1 million SARS-CoV-2 infections have been reported in Africa, of which 4.0 million cases are from South Africa [1]. Pregnant women are at similar risk as the general population for being infected by SARS-CoV-2 [2,3], albeit at higher risk of progressing to severe disease. In pregnant women COVID-19 may lead to adverse pregnancy outcomes, and the virus could transmit to the foetus or newborn [4,5]. Several clinical studies, analyses from national surveillance systems and systematic reviews have been published on COVID-19 in pregnant women. Nevertheless, studies evaluating the impact of SARS-CoV-2 infection on birth outcomes in African women are sparse, and mainly describe retrospective analyses or without contemporaneous control group of SARS-CoV-2 uninfected pregnant women [6-11].

SARS-CoV-2 infection during pregnancy has been associated with approximately a 2-fold increased risk of hospital admission, maternal admission to an intensive care unit (ICU) and invasive ventilation, compared with non-pregnant women [2,11-13]. Pregnant women with COVID-19 have also been reported to have a higher case fatality risk compared with non-infected pregnant women [11,14,15]. Risk-factors associated with severe COVID-19 in pregnant women are similar to those in non-pregnant adults [2,3,12,16-18].

SARS-CoV-2 infection during pregnancy has also been associated with a higher risk of preterm delivery, stillbirth, low-birthweight and lower Apgar scores at birth [2,8,15,17,19]. Nevertheless, since SARS-CoV-2 infection varies phenotypically from asymptomatic infection or mild respiratory illness to hospitalization with multiorgan failure and death [20], the severity of disease and the timing of infection during pregnancy may affect foetal outcome.

## METHODS

### Study design

We conducted a longitudinal cohort study of pregnant women investigated for SARS-CoV-2 infection during the antepartum period to assess any association of SARS-CoV-2 infection and adverse birth outcomes.

Pregnant women were screened for participation and enrolled from April 17, 2020 to September 18, 2020 which coincided with the first wave of the COVID-19 pandemic in South Africa. Follow-up for pregnancy outcomes continued until March 17, 2021. Women were enrolled at two public hospitals in the Johannesburg region where health care is provided at no cost to pregnant women and children: Chris Hani Baragwanath Academic Hospital (CHBAH), a tertiary care hospital, located in Soweto, and Rahima Moosa Mother and Child Hospital (RMMCH), a secondary district hospital located in Coronationville. The prevalence of HIV infection in antenatal attendees at the two facilities is approximately 28% at CHBAH and 19% at RMMCH. During the first wave of COVID-19 in South Africa, hospital protocols were established to triage pregnant women presenting with respiratory symptoms, where women managed as outpatients were seen in designated areas and those requiring hospitalization were admitted to isolation wards. Study-staff undertook active surveillance in the antenatal and hospital wards to identify pregnant women who presented with symptoms suggestive of COVID-19 (cough, sore throat, fever/feeling feverish, chest pain/shortness of breath, rhinitis, ageusia, anosmia, myalgia, arthralgia, headache or diarrhoea) for possible study enrolment and SARS-CoV-2 testing. In addition, asymptomatic women attending routine antenatal care were approached, enrolled, and tested for SARS-CoV-2 with maximum of 10 per day during weekdays. Women who consented to study participation were followed-up until delivery by regular telephonic contact and requested to contact the site when in labour. Study-staff also screened all women presenting in labour at the hospitals to identify those who had been enrolled antenatally. Women who delivered outside of the study-hospitals, or when no study-staff were on duty, had information on outcomes abstracted following review of their medical records or by interview.

The main outcome was to evaluate the association of SARS-CoV-2 infection diagnosed by nucleic acid amplification test (NAAT) in pregnant women with symptomatic illness (ie, COVID-19 group) and adverse birth out-

comes of spontaneous preterm delivery (<37 weeks gestational age), low-birthweight (<2500 g), very low-birthweight (<1500 g) or intrauterine foetal death. The referent group comprised of asymptomatic women at the time of enrolment with a negative NAAT result.

At enrolment, mid-turbinate nasal swabs were collected for investigation of SARS-CoV-2 infection by NAAT, as described [21]. NAAT was performed using the USA Centers for Diseases Control and Prevention Emergency use authorization assays to detect SARS-CoV-2 [22]. Samples were classified as positive for SARS-CoV-2 when both nucleocapsid targets (N1 and N2) were positive with a cycle threshold <40. Samples were classified as inconclusive if only a single target was detected.

Detailed information on demographics, health, pregnancy history and pregnancy complications were collected from all participants. At the time of delivery placentas were collected when possible. Placentas were placed in 10% buffered formalin and referred to the designated laboratory. Placental maternal and foetal surfaces were photographed before sampling according to standardised protocols and the macroscopic description recorded on a standardised template. Sections of the umbilical cord, membranes, and parenchyma were taken as recommended by the Amsterdam Placental Workshop Group Consensus protocols [23], and processed into paraffin embedded wax blocks. Subsequently, sections were cut, haematoxylin-eosin stained and produced for histological examination. Two dedicated pathologists (CW, WF), blinded to the SARS-CoV-2 infection status, examined the slides and reported on a standard template. The reported features were a summation of the macroscopic and microscopic features recorded. Additional details provided in the [Online Supplementary Document](#).

SARS-CoV-2 NAAT was also undertaken on placental tissue from women identified with antenatal SARS-CoV-2 infection and a random selection of women who had a negative NAAT result. Prior to nucleic acid extraction, paraffin-embedded placental tissues were de-paraffinized using 0.5% Tween-20 followed by heating in a 650W microwave for 45 seconds. The samples were subsequently centrifuged, and the solid wax disc was removed [24]. The placenta tissue was digested using 10mg/mL proteinase K (Sigma, Poole, UK) and mechanical disruption methods [25]. Total nucleic acids were extracted from lysed placental samples and nasal swabs using the Bioer automated extraction system together with MagaBio plus Virus DNA/RNA purification kit II as per manufactures' instructions (Hangzhou Bioer Technology Co. Ltd, China).

### Statistical considerations

Participants' categorical characteristics were described as percentages and were compared between women with COVID-19 and the control group or women with positive and negative SARS-CoV-2 NAAT by  $\chi^2$  or Fisher exact-test. Continuous variables were represented as mean or median and compared by Student's *t* test or Mann-Whitney test, respectively. *P* values <0.05 were considered significant.

Study outcomes were described per newborn, with multiple birth pregnancies evaluated independently. A variable combining newborns with either low-birthweight or born preterm was generated (preterm/low-birthweight). Placental histological characteristics were compared between women with positive and negative NAAT results on nasal swab and restricted to women who had a singleton birth.

Two sub-analyses were performed, i) comparison of outcomes between overall women with positive and negative NAAT results during pregnancy; ii) comparison of outcomes between symptomatic and asymptomatic women at enrolment stratified by NAAT positivity.

Odds ratios (OR), both crude and adjusted (aOR), and 95% confidence intervals (CI) were estimated by logistic regression. Covariates were selected a priori based on the literature, and these included: maternal HIV infection status, comorbid conditions, pregnancy-related complications, previous premature births, and time between diagnosis and delivery (7 categories overall, from <7 days to >90 days). Associations between a positive NAAT or COVID-19 and adverse outcomes were considered statistically significant if the 95% CI did not cross 1. Study data were collected and managed through Research Electronic Data Capture [24]. Analyses were performed using STATA 13.1 (StataCorp, College Station, Texas 77845 USA) and SAS/STAT 9.4 (SAS Institute Inc., Cary, North Carolina, USA).

## RESULTS

Overall, 793 women were tested for SARS-CoV-2 infection antenatally, including 275 (35%) who presented with symptoms suggestive of COVID-19. Twelve women in whom the NAAT result was inconclusive were excluded from the outcome analyses ([Figure 1](#)). Overall, SARS-CoV-2 infection was confirmed in 138 (17%) women, including 119 of 275 (43%) presenting with symptomatic illness (COVID-19 group) and 19 of 518

(4%) who were asymptomatic. Among the 643 women in whom SARS-CoV-2 was not identified, 493 (77%) were asymptomatic (ie, referent group).

At enrolment, the mean age was 31 years, the mean gestational age was 31.5 weeks, with no difference based on NAAT positivity on nasal swab or between women in the COVID-19 group or control group. Women with a positive NAAT result compared with those with a negative test were more likely to have comorbidities (25% vs 16%,  $P=0.015$ ) and pregnancy-related complications (55% vs 43%,  $P=0.023$ ); and similarly, so for women in the COVID-19 and the referent groups (Table 1).

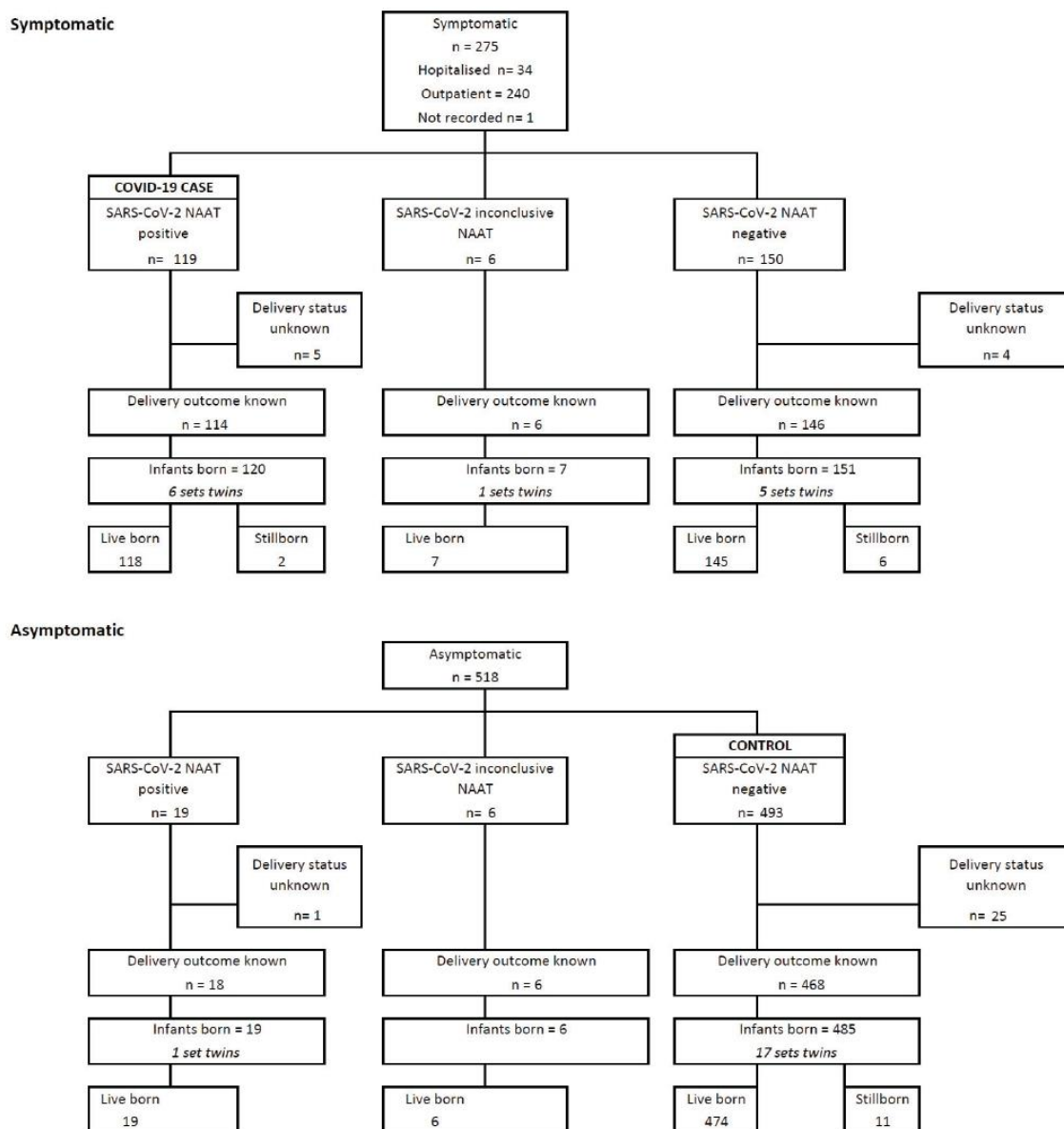


Figure 1. Participants' flow diagram accordingly to symptoms presentation antenatally at the time of study enrolment.

Table 1. Characteristics of women enrolled and tested for SARS-CoV-2 infection antenatally

|   | OVERALL<br>N = 793 | SARS-CoV-2 POSITIVE<br>N = 138 | SARS-CoV-2 NEGATIVE<br>N = 643 | SARS-CoV-2 INCONCLUSIVE<br>N = 12 | P-VALUE* | SYMPTOMATIC AND SARS-CoV-2 POSITIVE (COVID-19)<br>N = 119 | ASYMPTOMATIC AND SARS-CoV-2 NEGATIVE (CONTROL)<br>N = 493 | P-VALUE† |
|---|--------------------|--------------------------------|--------------------------------|-----------------------------------|----------|---|---|----------|
| Symptomatic at the time of enrolment                        |                    |                                |                                |                                   |          |   |   |          |
| Yes   | 275 (35)           | 119 (86)                       | 150 (23)                       | 6 (50)                            | <0.001   |   |   |          |
| No  | 518 (65)           | 19 (14)                        | 493 (77)                       | 6 (50)                            |          |   |   |          |
| Admitted for respiratory condition at the time of enrolment |                    |                                |                                |                                   |          |   |   |          |
| Yes   | 34 (4)             | 21 (15)                        | 13 (2)                         | 0 (0)                             | <0.001   | 21 (18)   | 0 (0)   |          |
| No  | 738 (96)           | 116 (85)                       | 630 (98)                       | 12 (100)                          |          | 97 (82)   | 493 (100)   |          |
| Missing   | 1                  | 1                              | 0                              | 0                                 |          | 1   | 0   |          |
| Mean age in year at enrolment, SD                           | 30.96, 8           | 31.8, 6.6                      | 30.7, 6.7                      | 33.8, 11.5                        | 0.08     | 31.8, 6.5   | 30.8, 6.6   | 0.16     |
| Race  |                    |                                |                                |                                   |          |   |   |          |
| Black   | 738 (96)           | 129 (93)                       | 620 (97)                       | 9 (75)                            | 0.09     | 112 (94)  | 479 (97)  | 0.16     |
| Asian   | 2 (0.3)            | 1 (1)                          | 1 (0.2)                        | 0 (0)                             |          | 1 (1)   | 1 (0.2)   |          |
| Coloured  | 26 (3)             | 6 (4)                          | 17 (3)                         | 3 (25)                            |          | 4 (3)   | 10 (2)  |          |
| Caucasian   | 4 (1)              | 1 (1)                          | 3 (0.5)                        | 0 (0)                             |          | 1 (1)   | 2 (0.4)   |          |
| Other   | 1 (0.1)            | 1 (1)                          | 0 (0)                          | 0 (0)                             |          | 1 (1)   | 0 (0)   |          |
| Missing   | 2                  | 0                              | 2                              | 0                                 |          | 0   | 1   |          |
| Mean gestational age in weeks at enrolment, SD              | 31.5, 6.9          | 31.0, 7.6                      | 31.6, 6.8                      | 33.4, 6.4                         | 0.39     | 30.9, 7.6   | 31.9, 6.6   | 0.14     |
| Gestational age in weeks at enrolment                       |                    |                                |                                |                                   |          |   |   |          |
| <34   | 405 (51)           | 73 (54)                        | 327 (51)                       | 5 (42)                            | 0.74     | 64 (55)   | 238 (48)  | 0.43     |
| 34-37   | 168 (21)           | 26 (19)                        | 141 (22)                       | 1 (8)                             |          | 22 (19)   | 111 (23)  |          |
| ≥37   | 214 (27)           | 37 (27)                        | 171 (27)                       | 6 (50)                            |          | 31 (26)   | 142 (29)  |          |
| <37   | 573 (73)           | 99 (73)                        | 468 (73)                       | 6 (50)                            |          | 86 (74)   | 349 (71)  |          |
| Missing   | 6                  | 2                              | 4                              | 0                                 |          | 2   | 2   |          |
| Median days between enrolment and delivery, IQR‡            | 30, 10-69 (739)    | 24, 6-72 (132)                 | 31, 12-69 (615)                | 15, 4-57                          | 0.11     | 25, 6-71 (114)  | 30, 12-66 (469)   | 0.38     |
| Multiple gestation pregnancy                                |                    |                                |                                |                                   |          |   |   |          |
| Yes   | 30 (4)             | 7 (5)                          | 22 (4)                         | 1 (8)                             | 0.35     | 6 (5)   | 17 (4)  | 0.42     |
| No  | 729 (96)           | 125 (95)                       | 593 (96)                       | 11 (92)                           |          | 108 (95)  | 452 (96)  |          |
| Missing   | 34                 | 6                              | 28                             | 0                                 |          | 5   | 24  |          |
| Previous premature births (<37 weeks)                       |                    |                                |                                |                                   |          |   |   |          |
| Yes   | 141 (20)           | 29 (22)                        | 109 (19)                       | 0 (0)                             | 0.022    | 11 (10)   | 24 (5)  | 0.09     |
| No  | 570 (80)           | 100 (78)                       | 463 (81)                       | 10 (100)                          |          | 100 (90)  | 415 (95)  |          |
| Missing   | 82                 | 9                              | 71                             | 2                                 |          | 8   | 54  |          |
| Previous miscarriages                                       |                    |                                |                                |                                   |          |   |   |          |
| Yes   | 30 (4)             | 7 (5)                          | 22 (4)                         | 3 (30)                            | 0.35     | 6 (5)   | 17 (4)  | 0.42     |
| No  | 729 (96)           | 125 (95)                       | 593 (96)                       | 7 (70)                            |          | 108 (95)  | 452 (96)  |          |
| Missing   | 34                 | 6                              | 28                             | 2                                 |          | 5   | 24  |          |
| Previous stillbirths  |                    |                                |                                |                                   | 0.82     |   |   | 0.79     |

Table 1. Continued

|                                  | OVERALL<br>N = 793 | SARS-COV-2 POS-<br>ITIVE N = 138 | SARS-COV-2 NEGATIVE<br>N = 643 | SARS-COV-2 INCON-<br>CLUSIVE N = 12 | P-VALUE* | SYMPTOMATIC AND SARS-COV-2<br>POSITIVE (COVID-19) N = 119 | ASYMPTOMATIC AND SARS-COV-2<br>NEGATIVE (CONTROL) N = 493 | P-VALUE† |
|----------------------------------|--------------------|----------------------------------|--------------------------------|-------------------------------------|----------|---|---|----------|
| Yes                              | 52 (7)             | 9 (7)                            | 43 (8)                         | 0 (0)                               |          | 8 (7)   | 35 (8)  |          |
| No                               | 656 (93)           | 120 (93)                         | 526 (92)                       | 10 (100)                            |          | 103 (93)  | 404 (92)  |          |
| Missing                          | 85                 | 9                                | 74                             | 2                                   |          | 8   | 54  |          |
| Comorbid conditions§             |                    |                                  |                                |                                     | 0.015    |   |   | 0.005    |
| Yes                              | 136 (18)           | 34 (25)                          | 102 (16)                       | 0 (0)                               |          | 29 (25)   | 68 (14)   |          |
| No                               | 640 (82)           | 102 (75)                         | 527 (84)                       | 11 (100)                            |          | 89 (75)   | 416 (86)  |          |
| Missing                          | 17                 | 2                                | 14                             | 1                                   |          | 1   | 9   |          |
| Living with HIV                  |                    |                                  |                                |                                     | 0.26     |   |   | 0.19     |
| Yes                              | 208 (27)           | 31 (23)                          | 176 (28)                       | 1 (8)                               |          | 25 (22)   | 134 (28)  |          |
| No                               | 569 (73)           | 103 (77)                         | 455 (72)                       | 11 (92)                             |          | 90 (78)   | 349 (72)  |          |
| Missing                          | 16                 | 4                                | 12                             | 0                                   |          | 4   | 10  |          |
| Syphilis serology                |                    |                                  |                                |                                     | 0.70     |   |   | 0.37     |
| Reactive                         | 11 (2)             | 1 (1)                            | 10 (2)                         | 0 (0)                               |          | 0 (0)   | 8 (2)   |          |
| Non-reactive                     | 689 (98)           | 121 (99)                         | 556 (98)                       | 12 (100)                            |          | 105 (100)   | 422 (98)  |          |
| Missing                          | 93                 | 16                               | 77                             | 0                                   |          | 14  | 63  |          |
| Influenza vaccinated             |                    |                                  |                                |                                     | 0.13     |   |   | 0.07     |
| Yes                              | 30 (4)             | 8 (6)                            | 20 (3)                         | 2 (17)                              |          | 7 (6)   | 13 (3)  |          |
| No                               | 743 (96)           | 126 (94)                         | 607 (97)                       | 10 (83)                             |          | 109 (94)  | 468 (97)  |          |
| Missing                          | 20                 | 4                                | 16                             | 0                                   |          | 3   | 12  |          |
| Tetanus toxoid vaccinated        |                    |                                  |                                |                                     | 0.61     |   |   | 0.12     |
| Yes                              | 447 (58)           | 75 (56)                          | 365 (58)                       | 7 (58)                              |          | 61 (53)   | 290 (61)  |          |
| No                               | 324 (42)           | 59 (44)                          | 260 (42)                       | 5 (42)                              |          | 55 (47)   | 189 (39)  |          |
| Missing                          | 22                 | 4                                | 18                             | 0                                   |          | 3   | 14  |          |
| Pregnancy related complications¶ |                    |                                  |                                |                                     | 0.023    |   |   | 0.040    |
| Yes                              | 343 (45)           | 74 (55)                          | 264 (43)                       | 5 (42)                              |          | 64 (55)   | 191 (41)  |          |
| No                               | 422 (55)           | 60 (45)                          | 355 (57)                       | 7 (58)                              |          | 52 (45)   | 280 (59)  |          |
| Missing                          | 28                 | 4                                | 24                             | 0                                   |          | 3   | 22  |          |

SD – standard deviation, IQR – interquartile range

\*P-values comparing women with a SARS-CoV-2 positive vs negative nucleic acid amplification test antenatally.

†P-values comparing symptomatic women with a SARS-CoV-2 positive nucleic acid amplification test vs asymptomatic women with a SARS-CoV-2 negative nucleic acid amplification test.

‡Numbers in parenthesis denote participants with available information.

§Comorbid conditions include: asthma, tuberculosis, chronic obstructive pulmonary disease/emphysema, other chronic lung disease, hypertension, cardiovascular disease, stroke, diabetes, anaemia, epilepsy, thyroid disease, cancer, malnutrition, obesity, renal failure, other organ failure.

¶Pregnancy related complication include: pregnancy induced hypertension, pre-eclampsia, eclampsia, HELLP (haemolysis, elevated liver enzymes, low platelet count), gestational diabetes, hyperemesis, trauma, pregnancy related infection, vaginal discharge, chorioamnionitis, maternal sepsis, maternal tachycardia, embolic disease, antepartum haemorrhage, placenta previa/accreta/increta, placental abruption, premature contractions, premature rupture of the membranes, oligohydramnios, polyhydramnios.

Results are n (%) unless stated otherwise. Percentages calculated excluding missing values.



Pregnancy outcomes were available for 132 women identified with SARS-CoV-2 infection and 614 who had a negative NAAT result, who gave birth to 139 and 636 newborns, respectively (Figure 1). Women with antepartum COVID-19 compared with the referent group were 1.66-times (95% CI = 1.02-2.71) more likely to deliver babies who were of low-birthweight (30% vs 21%) and 3.25-times (95% CI = 1.08-9.80) more likely of very low-birthweight (5% vs 2%). Similar observations were evident when restricting the analysis to women enrolled before 37 weeks gestational age (Table 2).

**Table 2.** Pregnancy outcomes in the control group and the COVID-19 group

|  | ALL STUDY PARTICIPANTS                         |  | OR (95% CI)                 | aOR* (95% CI)               |
|--|--|--|-----------------------------|-----------------------------|
|  | SYMPTOMATIC AND SARS-COV-2 POSITIVE (COVID-19) | ASYMPTOMATIC AND SARS-COV-2 NEGATIVE (CONTROL) |                             |                             |
|  | N (%)  | N (%)  |                             |                             |
|  | N = 120  | N = 485  |                             |                             |
| Live birth   | 118 (98)                                       | 474 (98)                                       | Reference, 0.73 (0.16-3.34) | Reference, 0.86 (0.17-4.36) |
| Foetal death   | 2 (2)  | 11 (2)   |                             |                             |
| <b>Live births overall</b>                                     | N = 118  | N = 474  |                             |                             |
| <b>Mode of delivery</b>  |  |  |                             |                             |
| Vaginal  | 48 (41)  | 206 (44)                                       | Reference                   | Reference                   |
| Caesarean-elective   | 25 (21)  | 101 (21)                                       | 1.06 (0.62-1.82)            | 1.27 (0.70-2.32)            |
| Caesarean-emergency  | 45 (38)  | 167 (35)                                       | 1.16 (0.73-1.82)            | 0.98 (0.61-1.59)            |
| <b>Apgar score – 5 min</b>                                     |  |  |                             |                             |
| 0-3  | 1 (1)  | 2 (0.5)  | 2.00 (0.18-22.32)           | 2.39 (0.20-28.35)           |
| 4-6  | 2 (2)  | 9 (2)  | 0.89 (0.19-4.18)            | 0.87 (0.17-4.31)            |
| 7-10   | 107 (97)                                       | 429 (97)                                       | Reference                   | Reference                   |
| <b>Respiratory distress</b>                                    |  |  |                             |                             |
| No   | 105 (91)                                       | 412 (89)                                       | Reference                   | Reference                   |
| Yes  | 11 (9)   | 450 (11)                                       | 0.86 (0.43-1.72)            | 0.70 (0.34-1.44)            |
| <b>Gestational age in weeks at delivery</b>                    |  |  |                             |                             |
| ≥37  | 87 (74)  | 390 (82)                                       | Reference                   | Reference                   |
| <37  | 30 (26)  | 84 (18)  | 1.60 (0.99-2.58)            | 1.48 (0.89-2.46)            |
| <b>Birthweight in grams</b>                                    |  |  |                             |                             |
| ≥2500  | 81 (70)  | 365 (79)                                       | Reference                   | Reference                   |
| <2500  | 34 (30)  | 95 (21)  | 1.61 (1.02-2.55)            | 1.66 (1.02-2.71)            |
| <1500  | 6 (5)  | 9 (2)  | 3.00 (1.04-8.68)            | 3.25 (1.08-9.80)            |
| <b>Preterm/Low-birthweight</b>                                 |  |  |                             |                             |
| No   | 74 (63)  | 351 (74)                                       | Reference                   | Reference                   |
| Yes  | 43 (37)  | 123 (26)                                       | 1.66 (1.08-2.54)            | 1.72 (1.08-2.68)            |
| <b>Live births, mothers enrolled at &lt;37 weeks gestation</b> | N = 85   | N = 335  |                             |                             |
| <b>Gestational age in weeks at delivery</b>                    |  |  |                             |                             |
| ≥37  | 55 (65)  | 251 (75)                                       | Reference                   | Reference                   |
| <37  | 30 (35)  | 84 (25)  | 1.63 (0.98-2.71)            | 1.48 (0.80-2.74)            |
| <b>Birthweight in grams</b>                                    |  |  |                             |                             |
| ≥2500  | 54 (64)  | 244 (75)                                       | Reference                   | Reference                   |
| <2500  | 30 (36)  | 81 (25)  | 1.67 (1.00-2.79)            | 1.52 (0.86-2.71)            |
| <1500  | 6 (7)  | 9 (3)  | 3.01 (1.03-8.82)            | 3.15 (0.99-10.00)           |
| <b>Preterm/Low-birthweight</b>                                 |  |  |                             |                             |
| No   | 46 (54)  | 226 (67)                                       | Reference                   | Reference                   |
| Yes  | 39 (46)  | 109 (33)                                       | 1.76 (1.08-2.85)            | 1.69 (0.96-2.97)            |
| <b>Live births, mothers enrolled at &lt;34 weeks gestation</b> | N = 63   | N = 229  |                             |                             |
| <b>Gestational age in weeks at delivery</b>                    |  |  |                             |                             |
| ≥37  | 39 (62)  | 162 (71)                                       | Reference                   | Reference                   |
| <37  | 24 (38)  | 67 (29)  | 1.49 (0.83-2.66)            | 1.31 (0.57-2.99)            |
| <34  | 13 (21)  | 32 (14)  | 1.69 (0.81-3.51)            | 1.05 (0.33-3.32)            |
| <b>Birthweight in grams</b>                                    |  |  |                             |                             |
| ≥2500  | 38 (60)  | 162 (73)                                       | Reference                   | Reference                   |
| <2500  | 25 (40)  | 60 (27)  | 1.78 (0.99-3.19)            | 1.53 (0.75-3.11)            |
| <1500  | 6 (10)   | 9 (4)  | 2.84 (0.95-8.47)            | 2.69 (0.74-9.82)            |
| <b>Preterm/Low-birthweight</b>                                 |  |  |                             |                             |
| No   | 32 (51)  | 146 (64)                                       | Reference                   | Reference                   |
| Yes  | 31 (49)  | 83 (36)  | 1.70 (0.97-2.99)            | 1.61 (0.77-3.37)            |

OR – odds ratio, aOR – adjusted odds ratio

\*Odds ratio adjusted for HIV status (infected, uninfected), comorbid conditions (yes, no), pregnancy related complications (yes, no), previous premature births (yes, no), and time between diagnosis and delivery (categorical variable); for aOR, records numbers are less due to missing data in variables used for adjustment. Odds ratio in bold are statistically significant.

Comparing women with SARS-CoV-2 infection (ie, COVID-19 and asymptomatic infections) to those in whom NAAT was negative at enrolment, the rate of foetal death (1% vs 3%) was similar. Women with SARS-CoV-2 infection, however, had higher odds (aOR=1.63, 95% CI=1.04-2.55) of delivering a low-birthweight baby (30% vs 22%) (Table 3).

**Table 3.** Pregnancy outcomes by maternal SARS-CoV-2 infection status antenatally

|  | ALL STUDY PARTICIPANTS |                     | OR (95% CI)       | aOR* (95% CI)     |
|--|------------------------|---------------------|-------------------|-------------------|
|  | SARS-CoV-2 POSITIVE    | SARS-CoV-2 NEGATIVE |                   |                   |
|  | N (%)                  | N (%)               |                   |                   |
|  | N= 139                 | N= 636              |                   |                   |
| Live birth   | 137 (99)               | 619 (97)            | Reference         | Reference         |
| Foetal death   | 2 (1)                  | 17 (3)              | 0.53 (0.12-2.33)  | 0.56 (0.12-2.64)  |
| <b>Live births overall</b>                                     |                        |                     |                   |                   |
|  | N= 137                 | N= 619              |                   |                   |
| <b>Mode of delivery</b>  |                        |                     |                   |                   |
| Vaginal  | 59 (43)                | 277 (45)            | Reference         | Reference         |
| Caesarean-elective   | 27 (20)                | 131 (21)            | 0.97 (0.59-1.60)  | 1.11 (0.64-1.93)  |
| Caesarean-emergency  | 51 (37)                | 211 (34)            | 1.13 (0.75-1.72)  | 0.96 (0.62-1.50)  |
| <b>Apgar score at 5 min</b>                                    |                        |                     |                   |                   |
| 0-3  | 1 (1)                  | 2 (0.4)             | 2.24 (0.20-24.87) | 2.28 (0.19-26.92) |
| 4-6  | 2 (2)                  | 11 (2)              | 0.81 (0.18-3.72)  | 0.92 (0.19-4.37)  |
| 7-10   | 126 (98)               | 564 (98)            | Reference         | Reference         |
| <b>Respiratory distress</b>                                    |                        |                     |                   |                   |
| No   | 122 (91)               | 534 (88)            | Reference         | Reference         |
| Yes  | 12 (9)                 | 71 (12)             | 0.74 (0.39-1.41)  | 0.61 (0.31-1.20)  |
| <b>Gestational age in weeks at delivery</b>                    |                        |                     |                   |                   |
| ≥37  | 99 (73)                | 489 (79)            | Reference         | Reference         |
| <37  | 37 (27)                | 128 (21)            | 1.43 (0.93-2.18)  | 1.28 (0.81-2.02)  |
| <b>Birth weight in grams</b>                                   |                        |                     |                   |                   |
| ≥2500  | 94 (70)                | 466 (78)            | Reference         | Reference         |
| <2500  | 40 (30)                | 133 (22)            | 1.49 (0.98-2.26)  | 1.63 (1.04-2.55)  |
| <1500  | 6 (4)                  | 18 (3)              | 1.65 (0.64-4.28)  | 1.81 (0.67-4.89)  |
| <b>Preterm/Low-birthweight</b>                                 |                        |                     |                   |                   |
| No   | 85 (62.5)              | 437 (71)            | Reference         | Reference         |
| Yes  | 51 (37.5)              | 182 (29)            | 1.44 (0.98-2.12)  | 1.44 (0.95-2.20)  |
| <b>Live births, mothers enrolled at &lt;37 weeks gestation</b> |                        |                     |                   |                   |
|  | N=98                   | N= 450              |                   |                   |
| <b>Gestational age in weeks at delivery</b>                    |                        |                     |                   |                   |
| ≥37  | 61 (62)                | 322 (72)            | Reference         | Reference         |
| <37  | 37 (38)                | 128 (28)            | 1.53 (0.97-2.41)  | 1.20 (0.68-2.12)  |
| <b>Birth weight in grams</b>                                   |                        |                     |                   |                   |
| ≥2500  | 62 (64)                | 322 (74)            | Reference         | Reference         |
| <2500  | 35 (36)                | 114 (26)            | 1.60 (1.00-2.54)  | 1.53 (0.90-2.59)  |
| <1500  | 6 (6)                  | 18 (4)              | 1.73 (0.66-4.54)  | 1.73 (0.61-4.89)  |
| <b>Preterm/Low birthweight</b>                                 |                        |                     |                   |                   |
| No   | 52 (53)                | 287 (64)            | Reference         | Reference         |
| Yes  | 46 (47)                | 163 (36)            | 1.56 (1.00-2.42)  | 1.34 (0.79-2.28)  |
| <b>Live births mothers enrolled at &lt;34 weeks gestation</b>  |                        |                     |                   |                   |
|  | N=72                   | N= 313              |                   |                   |
| <b>Gestational age in weeks at delivery</b>                    |                        |                     |                   |                   |
| ≥37  | 44 (61)                | 214 (68)            | Reference         | Reference         |
| <37  | 28 (39)                | 99 (32)             | 1.38 (0.81-2.34)  | 0.97 (0.45-2.09)  |
| <34  | 16 (22)                | 46 (15)             | 1.69 (0.88-3.26)  | 0.90 (0.32-2.52)  |
| <b>Birth weight in grams</b>                                   |                        |                     |                   |                   |
| ≥2500  | 44 (61)                | 220 (72)            | Reference         | Reference         |
| <2500  | 28 (39)                | 84 (28)             | 1.67 (0.97-2.85)  | 1.61 (0.84-3.08)  |
| <1500  | 6 (8)                  | 16 (5)              | 1.88 (0.69-5.06)  | 1.85 (0.78-5.85)  |
| <b>Preterm/Low birthweight</b>                                 |                        |                     |                   |                   |
| No   | 37 (51)                | 192 (61)            | Reference         | Reference         |
| Yes  | 35 (49)                | 121 (39)            | 1.50 (0.90-2.51)  | 1.26 (0.64-2.51)  |

OR – odds ratio, aOR – adjusted odds ratio

\*Odds ratio adjusted for HIV status (infected, uninfected), comorbid conditions (yes, no), pregnancy related complications (yes, no), previous premature births (yes, no), and time between diagnosis and delivery (categorical variable); for aOR, records numbers are less due to missing data in variables used for adjustment. Odds ratio in bold are statistically significant.

Among women with a negative NAAT result, those presenting with symptoms at enrolment had higher rates of premature birth (aOR=1.93, 95% CI=1.13-3.31) compared with asymptomatic women (Table S1 in the **Online Supplementary Document**). No difference in outcomes were detected comparing symptomatic and asymptomatic women at enrolment with a positive NAAT result, although only 19 women had no symptoms (Table S2 in the **Online Supplementary Document**).

Thirty-four of the women enrolled were hospitalised for a respiratory illness at the time of enrolment, of whom 21 (62%) had confirmed COVID-19. Of the women hospitalized with COVID-19, four (19%) were admitted to ICU, two (10%) required ventilation and one demised three days after hospitalization. Post-mortem autopsy confirmed COVID-19 pneumonia as the cause of death in the decedent. Of the 13 women hospitalised with a respiratory illness but tested negative for SARS-CoV-2 infection, none were admitted to ICU or died. One additional death occurred following surgical complications during delivery by Caesarean section in a woman with SARS-CoV-2 which occurred four months prior to delivery.

Placental tissue was available for histological assessment in 35 (31 with COVID-19) and 41 women with and without antenatal SARS-CoV-2 infection, respectively. A positive NAAT result was obtained in 28% (9/32) of the placentas from women with antenatal SARS-CoV-2 infection. The placentas from women with antenatal SARS-CoV-2 infection were more likely to have chorangioma (OR=3.40, 95% CI=1.18-9.84), although not significant in adjusted analysis (OR=3.01, 95% CI=0.99-9.17). Maternal vascular malperfusion was more frequently identified in women who tested negative for SARS-CoV-2 antenatally (aOR=0.28, 95% CI=0.09-0.89). There were no other histological differences in the placental tissue of women with and without antenatal documented SARS-CoV-2 infection (Table 4).

## DISCUSSION

In this prospective study, we report that antenatal SARS-CoV-2 infection mostly detected in the third trimester of pregnancy, was associated with 1.6-higher odds of having a low-birthweight newborn. A similar observation was evident when restricting the analysis to symptomatic infections, ie, COVID-19 cases. Although most of the participants in our study had mild disease, 21 of the SARS-CoV-2 infected women were hospitalized – among whom one died due to COVID-19 pneumonia.

A meta-analysis of studies up to June 30, 2021 reported that pregnant women with COVID-19 compared to those without had 1.69-higher odds of having a low-birthweight newborn (based on 3 studies with 807 and 1743 women, respectively), 1.86-higher odds of preterm birth (18 studies including 10 555 and 498 064 women, respectively), 1.46-higher odds of stillbirth (based on 6 studies with 8392 and 487 395 women, respectively) and 1.44-higher odds of 5th minute Apgar score of less than 7 (based on 4 studies with 2777 and 88909 women, respectively); although symptoms at the time of testing and timing of SARS-CoV-2 infection were not described for these outcomes [15].

As previously reported, we confirmed that SARS-CoV-2 could be associated with asymptomatic infections [21,26]. When looking at symptoms presentation the INTERCOVID Multinational Cohort Study, conducted in 18 countries among 706 pregnant women with and 1424 without SARS-CoV-2 infection at any stage of pregnancy or delivery, reported a trend for earlier gestational age at delivery in women with symptomatic compared with asymptomatic SARS-CoV-2 infection [8]. Also, in a Spanish population-based study even though no differences were found in the overall rate of adverse outcomes among SARS-CoV-2 infected and uninfected pregnant women; those with symptomatic COVID-19 had increased rates of preterm delivery (7.2% vs 16.9%) and intrapartum foetal distress (9.1% vs 19.2%) compared with SARS-CoV-2 uninfected women. Women with asymptomatic infection, however, had similar rates of preterm delivery compared with women who had not been infected by SARS-CoV-2 [27]. In contrast, the frequency of preterm birth (13%) among pregnant women in the USA with SARS-CoV-2 infection was similar in those presenting with symptoms (2315 women) compared with asymptomatic women (376 women) in unadjusted analyses [28]. Our study results underscore the importance to stratify SARS-CoV-2 infections into symptomatic (COVID-19) and asymptomatic illness and the contribution of symptoms due to other causes. Notably, we also observed a higher frequency of preterm deliveries among women with negative SARS-CoV-2 NAAT results in those with compared to those without respiratory symptoms, which could have been due to other respiratory pathogens infections. Notably, however, seasonal influenza virus was not circulating during the time period of our study [29].

There have been few reports on placental pathology in mothers who acquired SARS-CoV-2 during pregnancy describing a spectrum of features from normal tissue to diffuse inflammatory patterns in the intervillous space [30-32]. The lack of conformity in the period between acquiring the virus to delivery, maternal comorbidities

**Table 4.** Placenta histological features by maternal SARS-CoV-2 infection status

|   | SARS-CoV-2 NEGATIVE,<br>N = 41, n (%)† | SARS-CoV-2 NEGATIVE,<br>N = 41, n (%)† | OR (95% CI)       | aOR‡ (95% CI)     |
|---|--|--|-------------------|-------------------|
| <b>Chorioamnionitis</b>   |  |  |                   |                   |
| Absent  | 26 (74)                                | 28 (68)                                | Reference         | Reference         |
| Present   | 9 (26)                                 | 13 (32)                                | 0.75 (0.27-2.03)  | 0.64 (0.22-1.85)  |
| Chronic   | 2 (6)                                  | 1 (2)                                  | 2.15 (0.18-25.19) | 2.00 (0.16-24.21) |
| Acute   | 7 (20)                                 | 12 (29)                                | 0.63 (0.21-1.84)  | 0.53 (0.17-1.65)  |
| <b>Acute chorioamnionitis with maternal and foetal response</b> |  |  |                   |                   |
| Absent  | 4/7 (57)                               | 7/11 (64)                              | Reference         | Reference         |
| Present   | 3/7 (43)                               | 4/11 (36)                              | 1.31 (0.19-9.10)  | 1.17 (0.13-10.52) |
| <b>Funinitis</b>  |  |  |                   |                   |
| Absent  | 34 (97)                                | 38 (93)                                | Reference         | Reference         |
| Present   | 1 (3)                                  | 3 (7)                                  | 0.37 (0.04-3.75)  | 0.27 (0.02-2.98)  |
| <b>Chronic villitis</b>   |  |  |                   |                   |
| Absent  | 19 (54)                                | 25 (61)                                | Reference         | Reference         |
| Present   | 16 (46)                                | 16 (39)                                | 1.32 (0.53-3.28)  | 1.00 (0.38-2.65)  |
| High grade  | 7 (20)                                 | 10 (24)                                | 0.92 (0.30-2.87)  | 0.56 (0.16-1.96)  |
| Low grade   | 9 (26)                                 | 6 (15)                                 | 1.97 (0.60-6.51)  | 1.85 (0.52-6.60)  |
| <b>Histiocytic intervillitis</b>                                |  |  |                   |                   |
| Absent  | 35 (100)                               | 37 (90)                                | Reference         | Reference         |
| Present   | 0 (0)                                  | 4 (10)                                 | Na                | Na                |
| <b>Combination§</b>   |  |  |                   |                   |
| Absent  | 35 (100)                               | 40 (98)                                | Reference         | Reference         |
| Present   | 0 (0)                                  | 1 (2)                                  | Na                | Na                |
| <b>Meconium laden macrophages</b>                               |  |  |                   |                   |
| Absent  | 19 (54)                                | 22/40 (55)                             | Reference         | Reference         |
| Present   | 16 (46)                                | 18/40 (45)                             | 1.03 (0.41-2.56)  | 1.10 (0.42-2.89)  |
| <b>Maternal vascular malperfusion</b>                           |  |  |                   |                   |
| Absent  | 25 (71)                                | 21 (51)                                | Reference         | Reference         |
| Present   | 10 (29)                                | 20 (49)                                | 0.42 (0.16-1.09)  | 0.28 (0.09-0.89)  |
| <b>Foetal vascular malperfusion</b>                             |  |  |                   |                   |
| Absent  | 20/34 (59)                             | 25/40 (63)                             | Reference         | Reference         |
| Present   | 14/34 (41)                             | 15/40 (37)                             | 1.17 (0.46-2.97)  | 1.16 (0.43-3.10)  |
| Global  | 12/34 (35)                             | 14/40 (35)                             | 1.07 (0.41-2.83)  | 1.22 (0.44-3.34)  |
| Segmental   | 2/34 (6)                               | 1/40 (3)                               | 2.50 (0.21-29.60) | 0.62 (0.03-12.58) |
| <b>Foetal vascular malperfusion with cord abnormalities</b>     |  |  |                   |                   |
| Absent  | 10/14 (71)                             | 10/15 (67)                             | Reference         | Reference         |
| Present   | 4/14 (29)                              | 5/15 (33)                              | 0.80 (0.16-3.88)  | 0.68 (0.06-7.92)  |
| <b>Retroplacental hematoma</b>                                  |  |  |                   |                   |
| Absent  | 17 (49)                                | 20 (49)                                | Reference         | Reference         |
| Present   | 18 (51)                                | 21 (51)                                | 1.00 (0.41-2.49)  | 0.88 (0.34-2.32)  |
| <b>Foetal compromise</b>  |  |  |                   |                   |
| <b>Edematous villi</b>  |  |  |                   |                   |
| Absent  | 22/34 (65)                             | 29 (71)                                | Reference         | Reference         |
| Present   | 12/34 (35)                             | 12 (29)                                | 1.32 (0.50-3.49)  | 1.67 (0.59-4.71)  |
| <b>Increased foetal nucleated red blood cells</b>               |  |  |                   |                   |
| Absent  | 32/34 (94)                             | 40 (98)                                | Reference         | Reference         |
| Present   | 2/34 (6)                               | 1 (2)                                  | 2.50 (0.22-28.83) | 1.10 (0.06-19.35) |
| <b>Chorangiosis</b>   |  |  |                   |                   |
| Absent  | 20/34 (59)                             | 34 (83)                                | Reference         | Reference         |
| Present   | 14/34 (41)                             | 7 (17)                                 | 3.40 (1.18-9.84)  | 3.01 (0.99-9.17)  |

OR – odds ratio, aOR – adjusted odds ratio, Na – not applicable

\*Includes 4 asymptomatic women at the time of enrolment and 31 presenting with symptoms. SARS-CoV-2 nucleic acid amplification test was performed in 32 placentas and was positive in nine.

†Includes 30 asymptomatic women at the time of enrolment and 11 presenting with symptoms.

‡Odds ratio adjusted for HIV status (infected, uninfected), comorbid conditions (yes, no), pregnancy related complications (yes, no).

§Combination of high grade lymphocytic and histiocytic villitis and histiocytic intervillitis and diffuse perivillous fibrin. Odds ratio in bold are statistically significant.

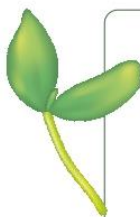
and variability in the laboratory methods used to confirm infection, however, limit interpretation of the current data. In our study, we identified higher prevalence of chorangiomas in the placentas of women with documented SARS-CoV-2 infection. Chorangiomas in a nonspecific adaptive placental response to decreased maternal oxygen tension, usually chronic or prolonged and has been previously described in the placentas from mothers with anaemia, diabetes, umbilical cord obstruction, or who smoke, or who live at very high altitudes [33,34]. It has also been reported among COVID-19 cases [35]. Maternal vascular malperfusion, although detected on both positive and negative NAAT groups, was more frequently identified in those women without history of SARS-CoV-2 infection. The women included in our study, done at two secondary and tertiary hospitals, had many other comorbidities including HIV infection. We found that >40% of the women had pregnancy-related complications, which can independently result in placental changes. The inclusion of a SARS-CoV-2 uninfected group is crucial to describe the specific effect that SARS-CoV-2 infection has on the placenta.

A limitation of our study was that antenatal testing was performed only once during pregnancy and we cannot exclude the possibility that women who had a negative NAAT result at time of enrolment may not have acquired an infection at a later stage. Also, since gestational sonar was not performed as part of the study and not done routinely, we relied on the date of the last menstrual period assessment documented in antenatal and medical notes to assess gestational age, which may lead to inaccurate gestational age staging [36]. Therefore, we used low and very low birthweight as a proxy for preterm birth. This was an observational study and therefore subject to potential biases and confounding effects, although we adjusted the outcome analyses for HIV infection status, other comorbid conditions and pregnancy-related complications, we did not evaluate the full impact of these variables.

Our study demonstrates that in a setting with high HIV infection prevalence and other comorbidities antenatal SARS-CoV-2 infection was associated with preterm delivery. Another study in South Africa, also during the first pandemic wave (May to July 2020), among women with high-risk pregnancies with COVID-19, found high rates (40%) of severe COVID-19 but no differences in disease severity or pregnancy outcomes in women living with HIV compared with pregnant women with other conditions [37]. A retrospective analysis of pregnant women from six African countries found that the frequencies of preterm births and low-birthweight infants were similar between 213 women with and 302 without COVID-19, however this cohort only included hospitalized women and so the control group was heterogeneous with a range of diseases that could have been associated with adverse pregnancy outcomes [11].

## CONCLUSIONS

More studies are needed from low- and middle-income countries to understand the possible impact of other comorbidities in pregnancy outcomes, since it has been suggested that pregnant women and their neonates from low- and middle-income countries are more vulnerable to adverse outcomes due to COVID-19 than women from high income countries [38]. Vaccination of pregnant women with COVID-19 vaccines has been shown to induce a good immune response, with efficient transplacental transfer of binding antibody to the foetus [39]. Also, COVID-19 vaccination of pregnant women reduces the risk of COVID-19 in the women and their young infants; and has also been reported to lower the risk of stillbirths [40]. Therefore, COVID-19 vaccination should be encouraged in pregnant women.



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**Ethics statement:** The study was approved by the University of the Witwatersrand Human Research Ethics Committee (181110 and 20313). To participate in the study, women had to provide written informed consent and be aged ≥18 years, or alternatively, if <18 years, consent signed by participants' partner/spouse or parent or other legally acceptable guardian, with assent co-signed by under-age mother.

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**Additional material:**

Online Supplementary Document

- 1 WHO. COVID-19 Dashboard. Geneva: World Health Organization, 2020. Available: <https://covid19.who.int/>. Accessed: 30 June 2022.
- 2 Allotey J, Stallings E, Bonet M, Yap M, Chatterjee S, Kew T, et al. Clinical manifestations, risk factors, and maternal and perinatal outcomes of coronavirus disease 2019 in pregnancy: living systematic review and meta-analysis. *BMJ*. 2020;370:m3320. Medline:32873575 doi:10.1136/bmj.m3320
- 3 Khalil A, Kalafat E, Benlioglu C, O'Brien P, Morris E, Draycott T, et al. SARS-CoV-2 infection in pregnancy: A systematic review and meta-analysis of clinical features and pregnancy outcomes. *EclinicalMedicine*. 2020;25:100446. Medline:32838230 doi:10.1016/j.eclinm.2020.100446
- 4 Dashraath P, Wong JIJ, Lim MXK, Lim LM, Li S, Biswas A, et al. Coronavirus disease 2019 (COVID-19) pandemic and pregnancy. *Am J Obstet Gynecol*. 2020;222:521-31. Medline:32217113 doi:10.1016/j.ajog.2020.03.021
- 5 Wastnedge EAN, Reynolds RM, van Boeckel SR, Stock SJ, Denison FC, Maybin JA, et al. Pregnancy and COVID-19. *Physiol Rev*. 2021;101:303-18. Medline:32969772 doi:10.1152/physrev.00024.2020
- 6 Basu JK, Chauke L, Cert M, Fetal M, Magoro T. Clinical Features and Outcomes of COVID-19 Infection among Pregnant Women in South Africa. *Int J MCH AIDS*. 2021;10:1-9. Medline:34262795
- 7 Hcini N, Maamri F, Picone O, Carod JF, Lambert V, Mathieu M, et al. Maternal, fetal and neonatal outcomes of large series of SARS-CoV-2 positive pregnancies in peripartum period: A single-center prospective comparative study. *Eur J Obstet Gynecol Reprod Biol*. 2021;257:11-8. Medline:33310656 doi:10.1016/j.ejogrb.2020.11.068
- 8 Villar J, Ariff S, Gunier RB, Thiruvengadam R, Rauch S, Kholin A, et al. Maternal and Neonatal Morbidity and Mortality Among Pregnant Women With and Without COVID-19 Infection: The INTERCOVID Multinational Cohort Study. *JAMA Pediatr*. 2021;175:817-26. Medline:33885740 doi:10.1001/jamapediatrics.2021.1050
- 9 Budhram S, Vannevel V, Botha T, Chauke L, Bhoora S, Balie GM, et al. Maternal characteristics and pregnancy outcomes of hospitalized pregnant women with SARS-CoV-2 infection in South Africa: An International Network of Obstetric Survey Systems-based cohort study. *Int J Gynaecol Obstet*. 2021;155:455-65. Medline:34499750 doi:10.1002/ijgo.13917
- 10 Osaikhuwuomwan J, Ezeanochie M, Uwagboe C, Ndukwu K, Yusuf S, Ande A. Clinical characteristics and outcomes for pregnant women diagnosed with COVID-19 disease at the University of Benin Teaching Hospital, Benin City, Nigeria. *Pan Afr Med J*. 2021;39:134. Medline:34527150 doi:10.11604/pamj.2021.39.134.27627
- 11 Nachega JB, Sam-Agudu NA, Machekano RN, Rosenthal PJ, Schell S, de Waard L, et al. Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) Infection and Pregnancy in Sub-Saharan Africa: A 6-Country Retrospective Cohort Analysis. *Clin Infect Dis*. 2022;ciac294. Medline:36130257 doi:10.1093/cid/ciac294
- 12 Zambrano LD, Ellington S, Strid P, Galang RR, Oduyebo T, Tong VI, et al. Update: Characteristics of Symptomatic Women of Reproductive Age with Laboratory-Confirmed SARS-CoV-2 Infection by Pregnancy Status - United States, January 22-October 3, 2020. *MMWR Morb Mortal Wkly Rep*. 2020;69:1641-7. Medline:33151921 doi:10.15585/mmwr.mm6944e3
- 13 Ciapponi A, Bardach A, Comande D, Berrueta M, Argento FJ, Rodriguez Cairolis F, et al. COVID-19 and pregnancy: An umbrella review of clinical presentation, vertical transmission, and maternal and perinatal outcomes. *PLoS One*. 2021;16:e0253974. Medline:34185807 doi:10.1371/journal.pone.0253974
- 14 Ko JY, DeSisto CL, Simeone RM, Ellington S, Galang RR, Oduyebo T, et al. Adverse Pregnancy Outcomes, Maternal Complications, and Severe Illness Among US Delivery Hospitalizations With and Without a Coronavirus Disease 2019 (COVID-19) Diagnosis. *Clin Infect Dis*. 2021;73 Suppl 1:S24-31. Medline:33977298 doi:10.1093/cid/ciab344
- 15 Pathirathna ML, Samarasekera BPP, Dasanayake TS, Saravanakumar P, Weerasekera I. Adverse Perinatal Outcomes in COVID-19 Infected Pregnant Women: A Systematic Review and Meta-Analysis. *Healthcare (Basel)*. 2022;10:203. Medline:35206820 doi:10.3390/healthcare10020203
- 16 Huntley BJF, Mulder IA, Di Mascio D, Vintzileos WS, Vintzileos AM, Berghella V, et al. Adverse Pregnancy Outcomes Among Individuals With and Without Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2): A Systematic Review and Meta-analysis. *Obstet Gynecol*. 2021;137:585-96. Medline:33706357 doi:10.1097/AOG.0000000000004320
- 17 Lassi ZS, Ana A, Das JK, Salam RA, Padhani ZA, Irfan O, et al. A systematic review and meta-analysis of data on pregnant women with confirmed COVID-19: Clinical presentation, and pregnancy and perinatal outcomes based on COVID-19 severity. *J Glob Health*. 2021;11:05018. Medline:34221361 doi:10.7189/jogh.11.05018
- 18 Molteni E, Astley CM, Ma W, Sudre CH, Magee LA, Murray B, et al. Symptoms and syndromes associated with SARS-CoV-2 infection and severity in pregnant women from two community cohorts. *Sci Rep*. 2021;11:6928. Medline:33767292 doi:10.1038/s41598-021-86452-3

- 19 Chinn J, Sedighim S, Kirby KA, Hohmann S, Hameed AB, Jolley J, et al. Characteristics and Outcomes of Women With COVID-19 Giving Birth at US Academic Centers During the COVID-19 Pandemic. *JAMA Netw Open*. 2021;4:e2120456. Medline:34379123 doi:10.1001/jamanetworkopen.2021.20456
- 20 Weiss P, Murdoch DR. Clinical course and mortality risk of severe COVID-19. *Lancet*. 2020;395:1014-5. Medline:32197108 doi:10.1016/S0140-6736(20)30633-4
- 21 Nunes MC, Baillie VL, Kwatra G, Bhikha S, Verwey C, Menezes C, et al. Severe Acute Respiratory Syndrome Coronavirus 2 Infection Among Healthcare Workers in South Africa: A Longitudinal Cohort Study. *Clin Infect Dis*. 2021;73:1896-900. Medline:33949670 doi:10.1093/cid/ciab398
- 22 Lu X, Wang L, Sakhivel SK, Whitaker B, Murray J, Kamili S, et al. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2. *Emerg Infect Dis*. 2020;26:1654-65. Medline:32396505 doi:10.3201/eid2608.201246
- 23 Khong TY, Mooney EE, Ariel I, Balmus NCM, Boyd TK, Brundler M-A, et al. Sampling and Definitions of Placental Lesions. *Arch Pathol Lab Med*. 2016;140:698-713. Medline:27223167 doi:10.5858/arpa.2015-0225-CC
- 24 Coombs NJ, Gough A, Primrose J. Optimisation of DNA and RNA extraction from archival formalin-fixed tissue. *Nucleic Acids Research*. 1999;27(16):e12. Medline:10454649 doi:10.1093/nar/27.16.e12
- 25 Diaz MH, Waller JL, Theodore MJ, Patel N, Wolff BJ, Benitez AJ, et al. Development and Implementation of Multiplex TaqMan Array Cards for Specimen Testing at Child Health and Mortality Prevention Surveillance Site Laboratories. *Clin Infect Dis*. 2019;69 Supplement\_4:S311-21. Medline:31598666 doi:10.1093/cid/ciz571
- 26 Croveto F, Crispi F, Llubra E, Figueras F, Gomez-Roig MD, Gratacos E. Seroprevalence and presentation of SARS-CoV-2 in pregnancy. *Lancet*. 2020;396:530-1. Medline:32771084 doi:10.1016/S0140-6736(20)31714-1
- 27 Croveto F, Crispi F, Llubra E, Pascal R, Larroya M, Trilla C, et al. Impact of Severe Acute Respiratory Syndrome Coronavirus 2 Infection on Pregnancy Outcomes: A Population-based Study. *Clin Infect Dis*. 2021;73:1768-75. Medline:33556958 doi:10.1093/cid/ciab104
- 28 Woodworth KR, Olsen EO, Neelam V, Lewis EL, Galang RR, Oduyebo T, et al. Birth and Infant Outcomes Following Laboratory-Confirmed SARS-CoV-2 Infection in Pregnancy - SET-NET, 16 Jurisdictions, March 29-October 14, 2020. *MMWR Morb Mortal Wkly Rep*. 2020;69:1635-40. Medline:33151917 doi:10.15585/mmwr.mm6944e2
- 29 Weekly respiratory pathogens report. Available: <https://www.nicd.ac.za/wp-content/uploads/2020/12/Weekly-Respiratory-Pathogens-Report-Week-50-2020.pdf>. Accessed: 30 June 2022.
- 30 Schwartz DA, Morotti D. Placental Pathology of COVID-19 with and without Fetal and Neonatal Infection: Trophoblast Necrosis and Chronic Histiocytic Intervillositis as Risk Factors for Transplacental Transmission of SARS-CoV-2. *Viruses*. 2020;12:1308. Medline:33203131 doi:10.3390/v12111308
- 31 Resta L, Vimercati A, Cazzato G, Mazzia G, Cicinelli E, Colagrande A, et al. SARS-CoV-2 and Placenta: New Insights and Perspectives. *Viruses*. 2021;13:723. Medline:33919284 doi:10.3390/v13050723
- 32 Edlow AG, Li JZ, Collier AY, Atyeo C, James KE, Boatman AA, et al. Assessment of Maternal and Neonatal SARS-CoV-2 Viral Load, Transplacental Antibody Transfer, and Placental Pathology in Pregnancies During the COVID-19 Pandemic. *JAMA Netw Open*. 2020;3:e2030455. Medline:33351086 doi:10.1001/jamanetworkopen.2020.30455
- 33 Daskalakis G, Marinopoulos S, Krielesi V, Papapanagiotou A, Papantoniou N, Mesogitis S, et al. Placental pathology in women with gestational diabetes. *Acta Obstet Gynecol Scand*. 2008;87:403-7. Medline:18382864 doi:10.1080/00016340801908783
- 34 Suzuki K, Itoh H, Kimura S, Sugihara K, Yaguchi C, Kobayashi Y, et al. Chorangiomas and placental oxygenation. *Congenit Anom (Kyoto)*. 2009;49:71-6. Medline:19489958 doi:10.1111/j.1741-4520.2009.00226.x
- 35 Shanes ED, Mithal LB, Otero S, Azad HA, Miller ES, Goldstein JA. Placental Pathology in COVID-19. *Am J Clin Pathol*. 2020;154:23-32. Medline:32441303 doi:10.1093/ajcp/aqaa089
- 36 Cutland CL, Cunningham M, Olugbosi M, Jones SA, Hugo A, Maharaj K, et al. Lessons learnt from enrolment and follow up of pregnant women and their infants in clinical trials in South Africa, a low-middle income country. *Vaccine*. 2015;33:6406-12. Medline:26409812 doi:10.1016/j.vaccine.2015.08.040
- 37 De Waard L, Langenegger E, Erasmus K, Van der Merwe T, Olivier SE, Du Toit N, et al. Maternal and neonatal outcomes of COVID-19 in a high-risk pregnant cohort with and without HIV. *S Afr Med J*. 2021;111:1174-80. Medline:34949304 doi:10.7196/SAMJ.2021.v111i12.15683
- 38 Gajbhiye RK, Sawant MS, Kuppasamy P, Surve S, Pasi A, Prusty RK, et al. Differential impact of COVID-19 in pregnant women from high-income countries and low- to middle-income countries: A systematic review and meta-analysis. *Int J Gynaecol Obstet*. 2021;155:48-56. Medline:34160059 doi:10.1002/ijgo.13793
- 39 Nunes MC, Madhi SA. COVID-19 vaccines in pregnancy. *Trends Mol Med*. 2022;28:662-80. Medline:35610123 doi:10.1016/j.molmed.2022.04.012
- 40 Prasad S, Kalafat E, Blakeway H, Townsend R, O'Brien P, Morris E, et al. Systematic review and meta-analysis of the effectiveness and perinatal outcomes of COVID-19 vaccination in pregnancy. *Nat Commun*. 2022;13:2414. Medline:35538060 doi:10.1038/s41467-022-30052-w

## Severe Acute Respiratory Syndrome Coronavirus 2 Infection Among Healthcare Workers in South Africa: A Longitudinal Cohort Study

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From April to September 2020, we investigated severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) infections in a cohort of 396 healthcare workers (HCWs) from 5 departments at Chris Hani Baragwanath Hospital, South Africa. Overall, 34.6% of HCWs had polymerase chain reaction–confirmed SARS-CoV-2 infection (132.1 [95% confidence interval, 111.8–156.2] infections per 1000 person-months); an additional 27 infections were identified by serology. HCWs in the internal medicine department had the highest rate of infection (61.7%). Among polymerase chain reaction–confirmed cases, 10.4% remained asymptomatic, 30.4% were presymptomatic, and 59.3% were symptomatic.

**Keywords.** COVID-19; SARS-CoV-2; hospital staff; healthcare workers; Africa.

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Healthcare workers (HCWs) are in the frontline of the coronavirus disease 2019 (COVID-19) outbreak response and consequently are at higher risk of infection by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) than the general population [1]. Understanding HCWs' SARS-CoV-2 exposure and risk of infection is critical for characterizing virus transmission patterns, risk factors for infection, and to inform the effectiveness of infection prevention and control practices. A modeling exercise from the United Kingdom suggested that weekly screening of HCWs for SARS-CoV-2 infection, irrespective of symptoms, could reduce transmission by 16%–23%, if results were available within 24 hours [2]. Studies from Europe reported rates of SARS-CoV-2 polymerase chain reaction (PCR) positivity in up to 24% of symptomatic and 7.1% of asymptomatic HCWs [3–5]. A cross-sectional study from Egypt among asymptomatic HCWs identified a 14.3% PCR positivity rate at the height of the pandemic in the country [6]. Paired serologic testing for SARS-CoV-2 antibodies, can supplement PCR testing. Cross-sectional seroprevalence surveys among HCWs in Europe and the United States during the first wave of the COVID-19 outbreak reported seropositivity prevalence between 4% and 24% [7–10]. Similarly, a study in Cape Town, South Africa, reported 10.4% seroprevalence among HCWs from pediatric facilities enrolled between May and July 2020 [11]. In the current study, longitudinal cohort surveillance of HCWs aimed to determine the incidence of SARS-CoV-2 infection and describe the clinical presentation thereof among HCWs at a large tertiary care hospital in South Africa, during the first COVID-19 wave.

### METHODS

#### Study Design

We enrolled HCWs across 5 departments, including internal medicine (IM), intensive care, pediatrics, obstetrics and gynecology, and the Vaccines and Infectious Diseases Analytics (VIDA) research unit, at Chris Hani Baragwanath Academic Hospital (CHBAH) in South Africa, Africa's largest hospital. Details of the epidemic progression and management of COVID-19 cases at CHBAH are outlined in the [Supplementary Materials](#). Enrollment occurred between 22 April and 19 June 2020, and the VIDA staff were enrolled until 24 July. The current analysis was censored to events occurring until 15 September 2020. Nasal midturbinate swab samples were collected weekly for PCR testing, irrespective of symptoms suggestive of COVID-19. Venous blood samples were collected at the time of enrollment and every 2 weeks thereafter. For the current analysis, serology testing was done on the blood samples obtained at enrollment and last study visit. HCWs who tested PCR positive completed a daily symptom log for the following 10 days. SARS-CoV-2–infected participants had repeated swab



samples obtained approximately every 2–4 days until  $\geq 2$  consecutive negative tests. Participants were classified accordingly to symptoms, as detailed in the [Supplementary Materials](#).

#### Laboratory Methods

Details are provided in the [Supplementary Materials](#). Reverse-transcriptase PCR results were classified as positive for SARS-CoV-2 when both the nucleocapsid genes (N1 and N2) were detected at a cycle threshold (Ct) value  $< 40$ . Results were classified as inconclusive if the Ct value was  $< 40$  for N1 or N2, but not both. Serum and plasma samples were tested using an in-house Luminex assay based on reactivity to the receptor-binding domain (RBD) of the SARS-CoV-2 spike protein. A value of 45 AU/mL was selected as the threshold indicative of SARS-CoV-2 exposure, based on the highest value of RBD immunoglobulin (Ig) G in samples collected before COVID-19.

#### Statistical Analysis

Additional details are provided in the [Supplementary Materials](#). The incidence of PCR-confirmed SARS-CoV-2 infection was calculated using Poisson regression models. The cumulative SARS-CoV-2 infection rate was estimated at the end of follow-up based on a positive PCR result or seroresponse. The association between risk of SARS-CoV-2 infection and participants' characteristics was estimated by means of univariate and multivariate generalized linear models. Significance was assessed based on the overlap of the 95% confidence intervals (CIs). Mean PCR Ct values at either diagnosis or lowest recorded value and lengths of infection were compared between participants with different symptoms, using Student *t* tests. Differences were considered significant at  $P \leq .05$ .

#### Ethical Considerations

The study was approved by the Human Research Ethics Committee of the University of the Witwatersrand (no. 200405). All study participants provided written informed consent.

## RESULTS

Overall, 396 HCWs were enrolled, including 167 (42.2%) from the IM department. The mean age (standard deviation [SD]) was 38.0 (9.4) years, 82.6% were female, and the majority were black Africans. Fifty-seven percent reported having  $\geq 1$  comorbid conditions, including 38.8% who were obese (body mass index  $> 30$  [calculated as weight in kilograms divided by height in meters squared]) and 13.2% with hypertension; [Supplementary Table 1](#). From 22 April to 15 September 2020, 137 (34.6%) HCWs were confirmed with PCR to be infected by SARS-CoV-2, for an incidence density of 132.1 cases (95% CI, 111.8–156.2) per 1000 person-months. The epidemic trajectory curve across the 5 departments overlapped ([Supplementary Figure 1](#)), with 115 (83.9%) PCR-confirmed cases detected between 15 June and 19 July 2020. The frequency of PCR-confirmed SARS-CoV-2

infection among HCWs from the IM department was 50.9% (85 of 167), with an incidence density of 203.0 cases (95% CI, 164.1–251.0) per 1000 person-months. Compared with IM, lower incidences were observed for staff from other departments (overall incidence, 22% [52 of 229]; 84.1 cases [95% CI, 64.1–110.4] per 1000 person-months), specifically from pediatrics and intensive care ([Supplementary Table 2](#)).

Of the HCWs with PCR-confirmed infections, 135 (98.5%) had information regarding symptoms at time of diagnosis, of whom 55 (40.7%) were asymptomatic and 80 (59.3%) had symptoms suggestive of COVID-19. Forty-one (74.6%) of the initially asymptomatic subsequently developed symptoms within 10 days after SARS-CoV-2 diagnosis (presymptomatic); thus, 14 (10.4%) were true asymptomatic cases. The frequency of the common symptoms is shown in [Supplementary Table 3](#). Symptomatic participants had lower N1 PCR Ct values at diagnosis (mean [SD], 24.2 [6.5]) and reached lower values (22.0 [4.8]) during the course of infection, compared with those who remained asymptomatic (28.9 [7.4] [ $P = .01$ ] and 28.8 [7.2] [ $P < .001$ ], respectively) or presymptomatic (28.0 [5.5] [ $P = .002$ ] and 25.1 [4.9] [ $P = .001$ ]). Accordingly, SARS-CoV-2 was persistently detected at Ct values  $\geq 30$  in a lower percentage of symptomatic (7.6%) than asymptomatic (42.9%) participants ( $P < .001$ ) ([Supplementary Table 4](#)).

Overall, SARS-CoV-2 was detected for a mean (SD) of 17.9 (8.8) days in nasal swab samples. Detection continued longer in symptomatic participants at the time of diagnosis (mean [SD] 18.9 [8.5] days) than in those remaining asymptomatic (13.0 [6.0] days;  $P = .04$ ) ([Supplementary Table 4](#) and [Supplementary Figure 2](#)).

Blood samples for serology testing were available from 395 (99.7%) HCWs at the time of enrollment, and 4 were seropositive but PCR negative for SARS-CoV-2. Despite being seropositive at enrollment, 2 (50%) subsequently had SARS-CoV-2 identified by PCR 24 and 69 days after enrollment. There were 135 PCR-confirmed SARS-CoV-2 cases evaluable for seroresponse, and all but 3 (2.2%) demonstrated seroresponse  $> 30$  days after diagnosis. In addition, 27 (12.8%) of the 211 HCWs who did not have a positive PCR result and underwent serology testing at the end of the follow-up demonstrated a seroresponse. The concordance between PCR positivity and seroresponse was 91.3% ( $\kappa = 0.82$ ).

Overall, 166 (41.9%) HCWs had evidence of SARS-CoV-2 infection, including 103 (61.7%) from IM and 63 (27.5%) working in other departments. In univariate analysis, working in the IM department, being a nurse, black African, or female, age  $> 38$  years, being hypertensive or obese, and taking public transport to work were associated with increased risk of SARS-CoV-2 infection. Conversely, receipt of influenza vaccine and smoking were associated with decreased risk. In multivariate analysis, however, only working in the IM department was associated with increased risk, with participants from other departments having an adjusted odds ratio of 0.29 (95% CI, .17–.49); [Table 1](#).

**Table 1. Characteristics of Healthcare Workers Enrolled in the Study and Risk of Developing Severe Acute Respiratory Syndrome Coronavirus 2 Infection Detected With Polymerase Chain Reaction or Serology**

| Characteristic                                 | HCWs, No. (%)        |                         | OR (95% CI)      |                 |
|--|----------------------|-------------------------|------------------|-----------------|
|  | SARS-CoV-2 (n = 166) | No SARS-CoV-2 (n = 230) | Unadjusted       | Adjusted        |
| <b>Department</b>                              |                      |                         |                  |                 |
| IM   | 103 (62.1)           | 64 (27.8)               | Reference        | Reference       |
| Pediatrics                                     | 31 (18.7)            | 62 (27.0)               | 0.31 (.18–53)    | ...             |
| Intensive care                                 | 11 (6.6)             | 38 (16.5)               | 0.18 (.09–38)    | ...             |
| Obstetrics                                     | 3 (1.8)              | 20 (8.7)                | 0.09 (.03–33)    | ...             |
| VIDA   | 18 (10.8)            | 46 (20.0)               | 0.24 (.13–46)    | ...             |
| Other than IM                                  | 63 (38.0)            | 166 (72.2)              | 0.24 (.15–36)    | 0.29 (.17–48)   |
| <b>Job category</b>                            |                      |                         |                  |                 |
| Nurse  | 111 (66.9)           | 82 (35.7)               | Reference        | Reference       |
| Physician                                      | 36 (21.7)            | 96 (41.7)               | 0.28 (.17–45)    | ...             |
| Paramedical                                    | 1 (0.6)              | 6 (2.6)                 | 0.12 (.01–1.04)  | ...             |
| VIDA clinical staff                            | 16 (9.6)             | 26 (11.3)               | 0.45 (.23–90)    | ...             |
| VIDA laboratory staff                          | 2 (1.2)              | 20 (8.7)                | 0.07 (.02–32)    | ...             |
| Other than nurse                               | 55 (33.1)            | 148 (64.4)              | 0.27 (.18–42)    | 0.72 (.37–1.42) |
| <b>Race</b>                                    |                      |                         |                  |                 |
| Black African                                  | 139 (83.7)           | 140 (60.9)              | Reference        | Reference       |
| Asian  | 16 (9.6)             | 41 (17.8)               | 0.39 (.21–73)    | ...             |
| White  | 10 (6.0)             | 39 (17.0)               | 0.26 (.12–54)    | ...             |
| Other  | 1 (0.6)              | 10 (4.4)                | 0.10 (.01–80)    | ...             |
| Other than black African                       | 27 (16.3)            | 90 (39.1)               | 0.30 (.19–49)    | 0.62 (.30–1.26) |
| <b>Sex</b>                                     |                      |                         |                  |                 |
| Female   | 148 (89.2)           | 179 (77)                | Reference        | Reference       |
| Male   | 18 (10.8)            | 51 (22.2)               | 0.43 (.24–76)    | 0.87 (.44–1.72) |
| <b>Age</b>                                     |                      |                         |                  |                 |
| <38 y  | 71 (42.8)            | 143 (62.2)              | Reference        | Reference       |
| ≥38 y  | 95 (57.2)            | 87 (37.8)               | 2.20 (1.46–3.30) | 1.25 (.75–2.10) |
| <b>Transport to work</b>                       |                      |                         |                  |                 |
| Public transport                               | 78 (47.0)            | 61 (26.5)               | Reference        | Reference       |
| Private car                                    | 84 (50.6)            | 166 (72.2)              | 0.40 (.26–61)    | ...             |
| Other  | 4 (2.4)              | 3 (1.3)                 | 1.04 (.22–4.83)  | ...             |
| Other than public transport                    | 88 (53.0)            | 169 (73.5)              | 0.41 (.27–62)    | 0.80 (.48–1.35) |
| <b>Smoking<sup>a</sup></b>                     |                      |                         |                  |                 |
| No   | 159 (95.8)           | 197 (85.7)              | Reference        | Reference       |
| Yes  | 7 (4.2)              | 33 (14.3)               | 0.26 (.11–61)    | 0.47 (.17–1.19) |
| <b>Influenza vaccination in current season</b> |                      |                         |                  |                 |
| No   | 96 (57.8)            | 96 (41.7)               | Reference        | Reference       |
| Yes  | 70 (42.2)            | 134 (58.3)              | 0.52 (.35–78)    | 1.24 (.73–2.08) |
| <b>≥1 Comorbid condition<sup>b</sup></b>       |                      |                         |                  |                 |
| No   | 63 (38.0)            | 106 (46.3)              | Reference        | ...             |
| Yes  | 103 (62.1)           | 123 (53.7)              | 1.41 (.94–2.12)  | ...             |
| BMI >30 <sup>c</sup>                           | 75 (45.7)            | 77 (33.8)               | 1.65 (1.09–2.49) | 0.97 (.59–1.59) |
| Hypertension                                   | 29 (17.5)            | 23 (10.0)               | 1.90 (1.05–3.41) | 1.04 (.52–2.06) |
| Asthma   | 10 (6.0)             | 20 (8.7)                | 0.67 (.30–1.47)  | ...             |
| HIV  | 11 (6.6)             | 10 (4.4)                | 1.55 (.64–3.75)  | ...             |
| Diabetes                                       | 5 (3.0)              | 4 (1.8)                 | 1.75 (.46–6.61)  | ...             |
| Sinusitis or allergy                           | 1 (0.6)              | 4 (1.8)                 | 0.34 (.04–3.08)  | ...             |
| Tuberculosis                                   | 0                    | 4 (1.8)                 | NS               | ...             |
| Cardiac disease                                | 0                    | 3 (1.3)                 | NS               | ...             |
| Pregnancy                                      | 0                    | 2 (0.9)                 | NS               | ...             |
| Other  | 2 (1.1)              | 8 (3.7)                 | 0.34 (.07–1.61)  | ...             |

Abbreviations: BMI, body mass index; CI, confidence interval; HCWs, healthcare workers; HIV, human immunodeficiency virus; IM, internal medicine; NS, not significant; OR, odds ratio; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; VIDA, Vaccines and Infectious Diseases Analytics.

<sup>a</sup>Active or previous smoker.

<sup>b</sup>For individual comorbid conditions, the reference is absence of that particular condition.

<sup>c</sup>BMI calculated as weight in kilograms divided by height in meters squared.

## DISCUSSION

In this longitudinal study, we evaluated the risk of SARS-CoV-2 infection at a large academic hospital in South Africa from the end of April through mid-September 2020. The rate of PCR-confirmed SARS-CoV-2 infection across the study population was 34.6%, which increased to 41.9% when including HCWs who were seropositive at enrollment or had a seroresponse during the study.

Although multiple studies have investigated SARS-CoV-2 infection among HCWs and suggested that exposure to patients with COVID-19 poses increased risk, the rates identified in our study are higher than those previously described in Europe and the United States using either PCR testing or serology surveys (4%–24%) [3, 4, 7–10]. At CHBAH, new strategies were developed to accommodate the unexpected increase in number of patients, including establishing specific wards for suspected and confirmed COVID-19 cases. The majority, of these wards, however, had poor ventilation and lacked isolation cubicles for individual patient management. These characteristics, together with crowded wards, likely contributed to these HCWs being at greater risk of SARS-CoV-2 infection than those in studies reported from high-income countries, where hospitals have better resources to reduce the risk of infections. Moreover, owing to the global shortage of high-quality filtering facepiece respirators, alternate products were sourced. A preliminary study contemporaneous with ours evaluating 12 brands of KN95 masks available in South Africa found that none of the brands met stipulated safety requirements, including mask material filtration efficacy and passing a seal and a qualitative fitting test [12].

We identified a differential risk of SARS-CoV-2 infection, with HCWs from the IM wards having the highest rate of infection. This group was the one mostly exposed to patients with COVID-19; other reasons for the differential rate of infection might be accessibility to training, space, and type of personal protective equipment usage. Although in univariate analysis we observed varying risk associated with job category and demographic factors, in multivariate analysis only working in the IM department remained a significant risk factor.

We identified a 97.8% seroresponse rate using the RBD IgG assay among HCWs with PCR-confirmed SARS-CoV-2 infection, which is consistent with previous reports [10]. When the respiratory samples from the 3 participants who failed to mount an immune response were retested, all of them were positive for N1 and N2, and 2 participants had PCR-positive samples collected at different time points, confirming true PCR positivity. The lack of antibodies against RBD does not imply lack of protection from future infections, since participants might have produced antibodies against other targets and be protected by other components of the immune system. We identified 2 PCR-confirmed SARS-CoV-2 infections in participants with

detectable antibodies against RBD before diagnosis. These reinfections were detected in early June and July before a new SARS-CoV-2 variant was identified in South Africa [13]. The antibody threshold defined during our Luminex assay validation was based on the identification of specific RBD IgG in clinical specimens; it does not necessarily correspond to a correlate of protection required for functional immunity, and higher levels of antibodies may be required to prevent upper respiratory tract reinfections.

In our study, 40.1% of participants were asymptomatic at the time of diagnosis, and 10.4% did not experience any symptoms within 10 days of diagnosis. Asymptomatic SARS-CoV-2 infections had lower viral load and shorter shedding duration, compared with symptomatic infections. A limitation of our study, however, is that detection of viral RNA does not necessarily imply the presence of infectious viruses in the respiratory tract. Nevertheless, current data suggest that viable virus is not shed beyond 20 days after symptom onset, with the probability of detecting live virus significantly decreasing after 5 days [14, 15]. Other study limitations are discussed in the [Supplementary Materials](#).

Providing HCWs with data about their SARS-CoV-2 virus exposure is important, so they can protect themselves, their patients, colleagues, and families; protecting HCWs is of paramount importance to fight this epidemic. Longitudinal studies like ours are important to understand the crucial correlation of SARS-CoV-2 antibody levels and protection against reinfection and possibly implications for immunity against new viral variants. In a relatively well-resourced setting in Africa, we identified a very high force of infection, suggesting that it is likely to be even higher elsewhere in the continent.

## Supplementary Data

Supplementary materials are available at *Clinical Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

## Notes

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**Study group.** The Bara HCW Study Group included Firdose Nakwa (Department of Paediatrics and Child Health, Chris Hani Baragwanath Academic Hospital [CHBAH], Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa); Sarah Van Blydenstein, Michelle Venter, and Denasha Reddy (Department of Internal Medicine, CHBAH, Faculty of Health Sciences, University of the Witwatersrand); and Jeanine du Plessis, Matt Laubscher, Lara van der Merwe, Nkululeko

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## References

1. Nguyen LH, Drew DA, Graham MS, et al; COronavirus Pandemic Epidemiology Consortium. Risk of COVID-19 among front-line health-care workers and the general community: a prospective cohort study. *Lancet Public Health* **2020**; 5:e475–83.
2. Nicholas C Grassly, Marga Pons-Salort, Edward PK Parker, Peter J White et al. Role of testing in COVID-19 control. Imperial College London, **2020**. doi:10.25561/78439.
3. Hunter E, Price DA, Murphy E, et al. First experience of COVID-19 screening of health-care workers in England. *Lancet* **2020**; 395:e77–8.
4. Treibel TA, Manisty C, Burton M, et al. COVID-19: PCR screening of asymptomatic health-care workers at London hospital. *Lancet* **2020**; 395:1608–10.
5. Lombardi A, Consonni D, Carugno M, et al. Characteristics of 1573 healthcare workers who underwent nasopharyngeal swab testing for SARS-CoV-2 in Milan, Lombardy, Italy. *Clin Microbiol Infect* **2020**; 26:1413.e9–e13.
6. Abdelmoniem R, Fouad R, Shawky S, et al. SARS-CoV-2 infection among asymptomatic healthcare workers of the emergency department in a tertiary care facility. *J Clin Virol* **2021**; 134:104710.
7. Sims MD, Maine GN, Childers KL, et al. COVID-19 seropositivity and asymptomatic rates in healthcare workers are associated with job function and masking. *Clin Infect Dis* **2021**; 73(Suppl 2):S154–62.
8. Self WH, Tenforde MW, Stubblefield WB, et al; CDC COVID-19 Response Team; IVY Network. Seroprevalence of SARS-CoV-2 among frontline health care personnel in a multistate hospital network—13 academic medical centers, April–June 2020. *MMWR Morb Mortal Wkly Rep* **2020**; 69:1221–6.
9. Moscola J, Sembajwe G, Jarrett M, et al; Northwell Health COVID-19 Research Consortium. Prevalence of SARS-CoV-2 antibodies in health care personnel in the New York City Area. *JAMA* **2020**; 324:893–5.
10. Garcia-Basteiro AL, Moncunill G, Tortajada M, et al. Seroprevalence of antibodies against SARS-CoV-2 among health care workers in a large Spanish reference hospital. *Nat Commun* **2020**; 11:3500.
11. Goldblatt D, Johnson M, Falup-Pecurariu O, et al. Cross-sectional prevalence of SARS-CoV-2 antibodies in healthcare workers in paediatric facilities in eight countries. *J Hosp Infect* **2021**; 110:60–6.
12. Mottay L, Le Roux J, Perumal R, et al. KN95 filtering facepiece respirators distributed in South Africa fail safety testing protocols. *S Afr Med J* **2020**; 0:13162. doi:10.7196/SAMJ.2021.v111i3.15381.
13. Tegally H, Wilkinson E, Giovanette M, et al. Emergence and rapid spread of a new severe acute respiratory syndrome-related coronavirus 2 (SARS-CoV-2) lineage with multiple spike mutations in South Africa. medRxiv [Preprint]. December 12, 2020. Available from: <https://www.medrxiv.org/content/10.1101/2020.12.21.20248640v1>.
14. Wölfel R, Corman VM, Guggemos W, et al. Virological assessment of hospitalized patients with COVID-2019. *Nature* **2020**; 581:465–9.
15. van Kampen JJA, van de Vijver DAMC, Fraaij PLA, et al. Duration and key determinants of infectious virus shedding in hospitalized patients with coronavirus disease-2019 (COVID-19). *Nat Commun* **2021**; 12:267.

## Future

As mentioned in the abstract, I plan to continue my research on the burden of respiratory pathogens and evaluate the impact of vaccines and other preventive strategies.

In an ongoing collaboration with the pediatrics department of the Hospices Civils de Lyon (HCL), we have started utilizing routinely collected patient data to explore various questions related to respiratory syncytial virus (RSV) infection in children. In a recently published paper, we stratified the risk of infants under one year of age developing clinically severe RSV infection. For this, we conducted a retrospective observational study involving infants born between 2014 and 2018 at the HCL. Using machine learning algorithms, we developed a tool to guide targeted prevention. Our approach addressed the challenge posed by the rarity of severe RSV infections within a large cohort, offering a solution through rigorous machine learning algorithms and thoughtful selection of simple variables available during the maternity stay (Horvat et al. 2024).

For a different project, since France was one of the few countries to introduce Nirsevimab (a long-acting monoclonal antibody to prevent RSV-associated lower respiratory tract infection [LRTI] in young infants) in maternities in September 2023, this provided us the opportunity to study the impact of this intervention in real life. We performed an observational study among infants younger than six months born and hospitalized at the HCL during the 2023-2024 season. Using the screening method, we estimated the effectiveness of Nirsevimab against hospitalization. We found that during the 2023-2024 season, 83 infants younger than six months were hospitalized with RSV-associated LRTI. Compared with historical periods, these infants were older. Hospitalization rates for infants born during the period when immunization was available were lower than in previous seasons; hospitalization rate ratios were 0.45 (95% confidence interval [CI]: 0.33-0.62) in 2023-2024 compared with the pre-COVID-19 period and 0.53 (95%CI: 0.36-0.77) compared with the 2022-2023 season. Nirsevimab effectiveness was 78.3% (95%CI: 55.9-89.5) with a coverage of 79.3% in the HCL maternities. We now plan to continue monitoring the impact of Nirsevimab in the next season, especially on:

- Effectiveness into the second year of life.
- RSV-associated paediatric outpatient visits.
- All-cause lower respiratory tract infection.

We plan to use the French health insurance data warehouse (SNDS, Système national des données de santé) to assess the severity of seasonal influenza and healthcare utilization in patients unvaccinated and vaccinated against influenza. Seasonal influenza annually affects approximately one million individuals in France, causing around 9,000 deaths, 90% of which are in adults over 65 years old. Inactivated standard-dose (SD) influenza vaccines (15µg per strain) are available in France and recommended for older adults (65 years and older). The high-dose (HD) vaccine with 60µg of antigen per strain was recently recommended. It has been shown that in France, the HD formulation was associated with a 23% (95% CI: 8%-36%) lower rate of influenza-associated hospitalizations compared to SD during the 2021-2022 season. Studies from the USA and South America suggested that influenza vaccination attenuated adverse outcomes in patients hospitalized with laboratory-confirmed influenza; however, there are no studies on these effects in Europe. We plan to:

- Assess and compare the severity of the disease at the index influenza-associated hospitalization among vaccinated and unvaccinated older adults.
- Compare the demographic and clinical characteristics of older adults hospitalized with a diagnosis of viral influenza infection according to their vaccination status.
- Assess disease progression after an influenza-associated hospitalization by vaccination status.
- Assess the post-discharge outcomes and mortality by vaccination status.
- Assess severity and clinical outcomes in patients with an additional diagnosis of a respiratory infection (different aetiology) according to vaccination status.
- Explore the differences between patients who received HD vs SD influenza vaccines.

While the burden of influenza and RSV has been extensively described in the literature, the epidemiology and clinical impact of other common viruses that might cause disease in the human population, such as rhinovirus, human metapneumovirus, and parainfluenza viruses, are less well-characterized. Understanding the epidemiology of other respiratory viruses beyond influenza and RSV is particularly pertinent as we move away from the recent COVID-19 pandemic. In a different project, we aim to characterize the epidemiology of other respiratory viruses beyond influenza and RSV, discussing their contribution to the overall burden of respiratory illness and related healthcare utilization. This will include:

- Description of their seasonality pre and post-COVID-19 pandemic.
- Clinical burden compared with that of influenza and RSV, and associated key severity outcomes such as hospital length of stay and intensive care unit admission.

- Estimated excess hospitalization and mortality trends.
- Comparison of these across countries and regions when possible.

Different databases and analytical approaches will be used to address the objectives of this project. The different viruses that we will be able to study in each objective may vary depending on access and availability of data (e.g., laboratory results, viral surveillance, clinical outcomes) in the databases. Databases will therefore be reviewed in the initial exploratory phase to determine the feasibility for the proposed analyses, especially regarding fit for purpose of the dataset to address the different objectives.

A last component of my future research will be to investigate the phylogenetic and evolution of viruses responsible for widespread infectious respiratory diseases and the impact on disease severity and immune escape. The identification of novel viral variants causing infectious respiratory diseases is crucial for the development of effective preparedness measures against emerging threats. A simple, yet powerful, machine learning algorithm was developed for early and unbiased identification of SARS-CoV-2 variants through the analysis of genomic data from the spike protein alone. In this project, we will expand on these observations. Firstly, we will explore the mutational landscape of influenza viruses and its impact on severity and immune escape using the sequencing and clinical data from HCL patients. Then the existing algorithm will be adapted and trained with more complete SARS-CoV-2 dataset. Furthermore, we will advance the algorithm by integrating more sophisticated neural network architectures for the use in other respiratory viruses.

## References

- Dejnirattisai, Wanwisa, Jiandong Huo, Daming Zhou, Jiří Zahradník, Piyada Supasa, Chang Liu, Helen M.E. Duyvesteyn, et al. 2022. 'SARS-CoV-2 Omicron-B.1.1.529 Leads to Widespread Escape from Neutralizing Antibody Responses'. *Cell* 185 (3): 467-484.e15. <https://doi.org/10.1016/j.cell.2021.12.046>.
- Dhar, Nisha, Gaurav Kwatra, Marta C Nunes, Clare Cutland, Alane Izu, Raffael Nachbagauer, Florian Krammer, and Shabir A Madhi. 2020. 'Hemagglutinin Stalk Antibody Responses Following Trivalent Inactivated Influenza Vaccine Immunization of Pregnant Women and Association With Protection From Influenza Virus Illness'. *Clinical Infectious Diseases* 71 (4): 1072–79. <https://doi.org/10.1093/cid/ciz927>.
- Gomes, M. Gabriela M., Ricardo Águas, João S. Lopes, Marta C. Nunes, Carlota Rebelo, Paula Rodrigues, and Claudio J. Struchiner. 2012. 'How Host Heterogeneity Governs Tuberculosis Reinfection?' *Proceedings of the Royal Society B: Biological Sciences* 279 (1737): 2473–78. <https://doi.org/10.1098/rspb.2011.2712>.
- Horvat, Côme, Cécile Chauvel, Jean-Sebastien Casalegno, Mehdi Benchaib, Dominique Ploin, Marta C. Nunes, and on behalf of the VRS Study Group in Lyon. 2024. 'RSV Severe Infection Risk Stratification in a French 5-Year Birth Cohort Using Machine-Learning'. *Pediatric Infectious Disease Journal*, May. <https://doi.org/10.1097/INF.0000000000004375>.
- Izu, Alane, Marta C Nunes, Fatima Solomon, Vicky Baillie, Natali Serafin, Charl Verwey, David P Moore, et al. 2023. 'All-Cause and Pathogen-Specific Lower Respiratory Tract Infection Hospital Admissions in Children Younger than 5 Years during the COVID-19 Pandemic (2020–22) Compared with the Pre-Pandemic Period (2015–19) in South Africa: An Observational Study'. *The Lancet Infectious Diseases* 23 (9): 1031–41. [https://doi.org/10.1016/S1473-3099\(23\)00200-1](https://doi.org/10.1016/S1473-3099(23)00200-1).
- Jallow, Sabelle, Shabir A. Madhi, Richard Madimabe, Nosisa Sipambo, Avy Violari, Udai Kala, Karen Petersen, et al. 2017. 'Immunogenicity of 13-Valent Pneumococcal Conjugate Vaccine among Children with Underlying Medical Conditions'. *Vaccine* 35 (34): 4321–29. <https://doi.org/10.1016/j.vaccine.2017.06.081>.
- Li, You, Rachel M Reeves, Xin Wang, Quique Bassat, W Abdullah Brooks, Cheryl Cohen, David P Moore, et al. 2019. 'Global Patterns in Monthly Activity of Influenza Virus, Respiratory Syncytial Virus, Parainfluenza Virus, and Metapneumovirus: A Systematic Analysis'. *The Lancet Global Health* 7 (8): e1031–45. [https://doi.org/10.1016/S2214-109X\(19\)30264-5](https://doi.org/10.1016/S2214-109X(19)30264-5).
- Lin, Jane H.-C., Takahiro Takano, Gregory Arcuino, Xiaohai Wang, Furong Hu, Zbigniew Darzynkiewicz, Marta Nunes, Steven A. Goldman, and Maiken Nedergaard. 2007. 'Purinergic Signaling Regulates Neural Progenitor Cell Expansion and Neurogenesis'. *Developmental Biology* 302 (1): 356–66. <https://doi.org/10.1016/j.ydbio.2006.09.017>.
- Liu, Chang, Helen M. Ginn, Wanwisa Dejnirattisai, Piyada Supasa, Beibei Wang, Aekkachai Tuekprakhon, Rungtiwa Nutalai, et al. 2021. 'Reduced Neutralization of SARS-CoV-2 B.1.617 by Vaccine and Convalescent Serum'. *Cell* 184 (16): 4220-4236.e13. <https://doi.org/10.1016/j.cell.2021.06.020>.
- Liu, Hui, Bin Lu, David E. Tabor, Andrey Tovchigrechko, Deidre Wilkins, Hong Jin, Shabir A. Madhi, Nasiha Soofie, Mark T. Esser, and Marta C. Nunes. 2020. 'Characterization of Human Respiratory Syncytial Virus (RSV) Isolated from HIV-exposed-uninfected and HIV-unexposed Infants in South Africa during 2015-2017'. *Influenza and Other Respiratory Viruses* 14 (4): 403–11. <https://doi.org/10.1111/irv.12727>.
- Lopez-Rubio, Jose Juan, Alisson M. Gontijo, Marta C. Nunes, Neha Issar, Rosaura Hernandez Rivas, and Artur Scherf. 2007. '5' Flanking Region of *Var* Genes Nucleate Histone Modification Patterns Linked to Phenotypic Inheritance of Virulence Traits in Malaria Parasites'. *Molecular Microbiology* 66 (6): 1296–1305. <https://doi.org/10.1111/j.1365-2958.2007.06009.x>.



- Madhi, Shabir A., Clare L. Cutland, Locadiah Kuwanda, Adriana Weinberg, Andrea Hugo, Stephanie Jones, Peter V. Adrian, et al. 2014. 'Influenza Vaccination of Pregnant Women and Protection of Their Infants'. *New England Journal of Medicine* 371 (10): 918–31. <https://doi.org/10.1056/NEJMoa1401480>.
- Mbotwe-Sibanda, Sthembile, Gaurav Kwatra, Shabir A Madhi, and Marta C Nunes. 2024. 'Post-Acute Sequelae of SARS-CoV-2 Infection in Health Care Workers from South Africa'. *Oxford Open Immunology* 5 (1): iqae001. <https://doi.org/10.1093/oxfimm/iqae001>.
- Mutsaerts, Eleonora, Shabir A. Madhi, Clare L. Cutland, Stephanie Jones, Andrea Hugo, Siobhan Trenor, Florette K. Treurnicht, Kerstin Klipstein-Grobusch, Adriana Weinberg, and Marta C. Nunes. 2016. 'Influenza Vaccination of Pregnant Women Protects Them over Two Consecutive Influenza Seasons in a Randomized Controlled Trial'. *Expert Review of Vaccines* 15 (8): 1055–62. <https://doi.org/10.1080/14760584.2016.1192473>.
- Nunes, Marta C, Vicky L Baillie, Gaurav Kwatra, Sutika Bhikha, Charl Verwey, Colin Menezes, Clare L Cutland, et al. 2021. 'Severe Acute Respiratory Syndrome Coronavirus 2 Infection Among Healthcare Workers in South Africa: A Longitudinal Cohort Study'. *Clinical Infectious Diseases* 73 (10): 1896–1900. <https://doi.org/10.1093/cid/ciab398>.
- Nunes, Marta C., Clare L. Cutland, Bonnie Dighero, Janie Bate, Stephanie Jones, Andrea Hugo, Nadia Van Niekerk, et al. 2015. 'Kinetics of Hemagglutination-Inhibiting Antibodies Following Maternal Influenza Vaccination Among Mothers With and Those Without HIV Infection and Their Infants'. *Journal of Infectious Diseases* 212 (12): 1976–87. <https://doi.org/10.1093/infdis/jiv339>.
- Nunes, Marta C, Clare L Cutland, Stephanie Jones, Sarah Downs, Adriana Weinberg, Justin R Ortiz, Kathleen M Neuzil, Eric A F Simões, Keith P Klugman, and Shabir A Madhi. 2017. 'Efficacy of Maternal Influenza Vaccination Against All-Cause Lower Respiratory Tract Infection Hospitalizations in Young Infants: Results From a Randomized Controlled Trial'. *Clinical Infectious Diseases* 65 (7): 1066–71. <https://doi.org/10.1093/cid/cix497>.
- Nunes, Marta C., Clare L. Cutland, Stephanie Jones, Andrea Hugo, Richard Madimabe, Eric A. F. Simões, Adriana Weinberg, Shabir A. Madhi, and for the Maternal Flu Trial Team. 2016. 'Duration of Infant Protection Against Influenza Illness Conferred by Maternal Immunization: Secondary Analysis of a Randomized Clinical Trial'. *JAMA Pediatrics* 170 (9): 840. <https://doi.org/10.1001/jamapediatrics.2016.0921>.
- Nunes, Marta C., Clare L. Cutland, Keith P. Klugman, and Shabir A. Madhi. 2021. 'Pneumococcal Conjugate Vaccine Protection against Coronavirus-Associated Pneumonia Hospitalization in Children Living with and without HIV'. Edited by Julian Parkhill. *mBio* 12 (1): e02347-20. <https://doi.org/10.1128/mBio.02347-20>.
- Nunes, Marta C., Clare L. Cutland, and Shabir A. Madhi. 2018. 'Influenza Vaccination during Pregnancy and Protection against Pertussis'. *New England Journal of Medicine* 378 (13): 1257–58. <https://doi.org/10.1056/NEJMc1705208>.
- Nunes, Marta C, Clare L Cutland, Andrew Moultrie, Stephanie Jones, Justin R Ortiz, Kathleen M Neuzil, Keith P Klugman, et al. 2020. 'Immunogenicity and Safety of Different Dosing Schedules of Trivalent Inactivated Influenza Vaccine in Pregnant Women with HIV: A Randomised Controlled Trial'. *The Lancet HIV* 7 (2): e91–103. [https://doi.org/10.1016/S2352-3018\(19\)30322-4](https://doi.org/10.1016/S2352-3018(19)30322-4).
- Nunes, Marta C., Sarah Downs, Stephanie Jones, Nadia Van Niekerk, Clare L. Cutland, and Shabir A. Madhi. 2016. '*Bordetella Pertussis* Infection in South African HIV-Infected and HIV-Uninfected Mother–Infant Dyads: A Longitudinal Cohort Study'. *Clinical Infectious Diseases* 63 (suppl 4): S174–80. <https://doi.org/10.1093/cid/ciw527>.
- Nunes, Marta C., J. P. Dean Goldring, Christian Doerig, and Artur Scherf. 2007. 'A Novel Protein Kinase Family in *Plasmodium Falciparum* Is Differentially Transcribed and Secreted to Various Cellular Compartments of the Host Cell'. *Molecular Microbiology* 63 (2): 391–403. <https://doi.org/10.1111/j.1365-2958.2006.05521.x>.

- Nunes, Marta C., Martin J. Hale, Sana Mahtab, Fikile C. Mabena, Noluthando Dlodlu, Vicky L. Baillie, Bukiwe N. Thwala, et al. 2022. 'Clinical Characteristics and Histopathology of COVID-19 Related Deaths in South African Adults'. Edited by Mohd Adnan. *PLOS ONE* 17 (1): e0262179. <https://doi.org/10.1371/journal.pone.0262179>.
- Nunes, Marta C., Stephanie A. Jones, Michelle J. Groome, Locadiah Kuwanda, Nadia Van Niekerk, Anne Von Gottberg, Linda De Gouveia, Peter V. Adrian, and Shabir A. Madhi. 2015. 'Acquisition of Streptococcus Pneumoniae in South African Children Vaccinated with 7-Valent Pneumococcal Conjugate Vaccine at 6, 14 and 40 Weeks of Age'. *Vaccine* 33 (5): 628–34. <https://doi.org/10.1016/j.vaccine.2014.12.023>.
- Nunes, Marta C., Stephanie Jones, Renate Strehlau, Vuyelwa Baba, Zanele Ditse, Kelly Da Silva, Lané Bothma, et al. 2022. 'Active Intrapartum SARS-CoV-2 Infection and Pregnancy Outcomes'. *American Journal of Perinatology* 39 (S 01): S42–48. <https://doi.org/10.1055/s-0042-1757274>.
- Nunes, Marta C., Stephanie Jones, Renate Strehlau, Vuyelwa Baba, Zanele Ditse, Kelly Da Silva, Lané Bothma, et al. 2022. 'Antepartum SARS-CoV-2 Infection and Adverse Birth Outcomes in South African Women'. *Journal of Global Health* 12 (December):05050. <https://doi.org/10.7189/jogh.12.05050>.
- Nunes, Marta C., Zachary Kuschner, Zeldá Rabede, Clare L. Cutland, Richard Madimabe, Locadiah Kuwanda, Keith P. Klugman, Peter V. Adrian, and Shabir A. Madhi. 2014. 'Polyomaviruses-Associated Respiratory Infections in HIV-Infected and HIV-Uninfected Children'. *Journal of Clinical Virology* 61 (4): 571–78. <https://doi.org/10.1016/j.jcv.2014.10.013>.
- Nunes, Marta C., Sthembile Mbotwe-Sibanda, Vicky L. Baillie, Gaurav Kwatra, Ricardo Aguas, Shabir A. Madhi, and on behalf of the Wits VIDA HCW Study Group. 2022. 'SARS-CoV-2 Omicron Symptomatic Infections in Previously Infected or Vaccinated South African Healthcare Workers'. *Vaccines* 10 (3): 459. <https://doi.org/10.3390/vaccines10030459>.
- Nunes, Marta C., Mami Okada, Christine Scheidig-Benatar, Brian M. Cooke, and Artur Scherf. 2010. 'Plasmodium Falciparum FIKK Kinase Members Target Distinct Components of the Erythrocyte Membrane'. Edited by Kirsten Nielsen. *PLoS ONE* 5 (7): e11747. <https://doi.org/10.1371/journal.pone.0011747>.
- Nunes, Marta C., Neeta Singh Roy, H. Michael Keyoung, Robert R. Goodman, Guy McKhann, Li Jiang, Jian Kang, Maiken Nedergaard, and Steven A. Goldman. 2003. 'Identification and Isolation of Multipotential Neural Progenitor Cells from the Subcortical White Matter of the Adult Human Brain'. *Nature Medicine* 9 (4): 439–47. <https://doi.org/10.1038/nm837>.
- Nunes, Marta C., Tinevimbo Shiri, Nadia Van Niekerk, Clare L. Cutland, Michelle J. Groome, Anthonet Koen, Anne Von Gottberg, et al. 2013. 'Acquisition of Streptococcus Pneumoniae in Pneumococcal Conjugate Vaccine-Naïve South African Children and Their Mothers'. *Pediatric Infectious Disease Journal* 32 (5): e192–205. <https://doi.org/10.1097/INF.0b013e31828683a3>.
- Nunes, Marta C., Nasiha Soofie, Sarah Downs, Naume Tebeila, Azwi Mudau, Linda De Gouveia, and Shabir A. Madhi. 2016. 'Comparing the Yield of Nasopharyngeal Swabs, Nasal Aspirates, and Induced Sputum for Detection of *Bordetella Pertussis* in Hospitalized Infants'. *Clinical Infectious Diseases* 63 (suppl 4): S181–86. <https://doi.org/10.1093/cid/ciw521>.
- Nunes, Marta C., Yvon Sterkers, Benoit Gamain, and Artur Scherf. 2008. 'Investigation of Host Factors Possibly Enhancing the Emergence of the Chondroitin Sulfate A-Binding Phenotype in Plasmodium Falciparum'. *Microbes and Infection* 10 (8): 928–32. <https://doi.org/10.1016/j.micinf.2008.05.006>.
- Nunes, Marta C., Amy Tamblyn, Lisa Jose, Mathapelo Ntsimane, Ntoetse Lerotholi, Charmaine Machimana, Ashleigh Taylor, Farzanah Laher, and Shabir A. Madhi. 2023. 'Immunogenicity of Tetanus, Diphtheria and Acellular Pertussis Vaccination among Pregnant Women Living with and without HIV'. *AIDS* 37 (15): 2305–10. <https://doi.org/10.1097/QAD.0000000000003731>.

- Nunes, Marta C., Anne Von Gottberg, Linda De Gouveia, Cheryl Cohen, Locadiah Kuwanda, Alan S. Karstaedt, Keith P. Klugman, and Shabir A. Madhi. 2011. 'Persistent High Burden of Invasive Pneumococcal Disease in South African HIV-Infected Adults in the Era of an Antiretroviral Treatment Program'. Edited by Philip Campbell Hill. *PLoS ONE* 6 (11): e27929. <https://doi.org/10.1371/journal.pone.0027929>.
- Nunes, Marta C, Anne Von Gottberg, Linda De Gouveia, Cheryl Cohen, David P Moore, Keith P Klugman, and Shabir A Madhi. 2011. 'The Impact of Antiretroviral Treatment on the Burden of Invasive Pneumococcal Disease in South African Children: A Time Series Analysis'. *AIDS* 25 (4): 453–62. <https://doi.org/10.1097/QAD.0b013e328341b7f1>.
- Nunes, Marta C, Sibongile Walaza, Susan Meiring, Heather J Zar, Gary Reubenson, Meredith McMorrow, Stefano Tempia, et al. 2022. 'Effectiveness of Influenza Vaccination of Pregnant Women for Prevention of Maternal and Early Infant Influenza-Associated Hospitalizations in South Africa: A Prospective Test-Negative Study'. *Open Forum Infectious Diseases* 9 (11): ofac552. <https://doi.org/10.1093/ofid/ofac552>.
- Nunes, Marta C., Adriana Weinberg, Clare L. Cutland, Stephanie Jones, David Wang, Bonnie Dighero-Kemp, Min Z. Levine, Niteen Wairagkar, and Shabir A. Madhi. 2018. 'Neutralization and Hemagglutination-Inhibition Antibodies Following Influenza Vaccination of HIV-Infected and HIV-Uninfected Pregnant Women'. Edited by Ray Borrow. *PLOS ONE* 13 (12): e0210124. <https://doi.org/10.1371/journal.pone.0210124>.
- Nunes, Marta Coelho, Soyeon Kim, Bret Zeldow, Avy Violari, Sylvia Dittmer, Haseena Cassim, Teena Thomas, et al. 2020. 'Streptococcus Pneumoniae Colonization in Pneumococcal Vaccine-Naïve Human Immunodeficiency Virus-Exposed Infected and -Uninfected South African Children'. *Medicine* 99 (9): e19353. <https://doi.org/10.1097/MD.00000000000019353>.
- Nunes, M. C., and A. Scherf. 2007. 'Plasmodium Falciparum during Pregnancy: A Puzzling Parasite Tissue Adhesion Tropism'. *Parasitology* 134 (13): 1863–69. <https://doi.org/10.1017/S0031182007000133>.
- Soofie, Nasiha, Marta C. Nunes, Prudence Kgagudi, Nadia Van Niekerk, Tselane Makgobo, Yasmeen Agosti, Cleopas Hwinya, Jayani Pathirana, and Shabir A. Madhi. 2016. 'The Burden of Pertussis Hospitalization in HIV-Exposed and HIV-Unexposed South African Infants'. *Clinical Infectious Diseases* 63 (suppl 4): S165–73. <https://doi.org/10.1093/cid/ciw545>.
- Van Noort, Sander P., Marta C. Nunes, Gareth D. Weedall, Lars Hviid, and M. Gabriela M. Gomes. 2010. 'Immune Selection and Within-Host Competition Can Structure the Repertoire of Variant Surface Antigens in Plasmodium Falciparum - A Mathematical Model'. Edited by James G. Beeson. *PLoS ONE* 5 (3): e9778. <https://doi.org/10.1371/journal.pone.0009778>.
- Viebig, Nicola K., Marta C. Nunes, Artur Scherf, and Benoit Gamain. 2006. 'The Human Placental Derived BeWo Cell Line: A Useful Model for Selecting Plasmodium Falciparum CSA-Binding Parasites'. *Experimental Parasitology* 112 (2): 121–25. <https://doi.org/10.1016/j.exppara.2005.09.007>.
- Wang, Xin, You Li, Maria Deloria-Knoll, Shabir A Madhi, Cheryl Cohen, Asad Ali, Sudha Basnet, et al. 2021. 'Global Burden of Acute Lower Respiratory Infection Associated with Human Metapneumovirus in Children under 5 Years in 2018: A Systematic Review and Modelling Study'. *The Lancet Global Health* 9 (1): e33–43. [https://doi.org/10.1016/S2214-109X\(20\)30393-4](https://doi.org/10.1016/S2214-109X(20)30393-4).
- Wang, Xin, You Li, Ting Shi, Louis J Bont, Helen Y Chu, Heather J Zar, Bhanu Wahi-Singh, et al. 2024. 'Global Disease Burden of and Risk Factors for Acute Lower Respiratory Infections Caused by Respiratory Syncytial Virus in Preterm Infants and Young Children in 2019: A Systematic Review and Meta-Analysis of Aggregated and Individual Participant Data'. *The Lancet* 403 (10433): 1241–53. [https://doi.org/10.1016/S0140-6736\(24\)00138-7](https://doi.org/10.1016/S0140-6736(24)00138-7).

- Wilkins, Deidre, Annefleure C Langedijk, Robert Jan Lebbink, Christopher Morehouse, Michael E Abram, Bahar Ahani, Anastasia A Aksyuk, et al. 2023. 'Nirsevimab Binding-Site Conservation in Respiratory Syncytial Virus Fusion Glycoprotein Worldwide between 1956 and 2021: An Analysis of Observational Study Sequencing Data'. *The Lancet Infectious Diseases* 23 (7): 856–66. [https://doi.org/10.1016/S1473-3099\(23\)00062-2](https://doi.org/10.1016/S1473-3099(23)00062-2).
- Windrem, Martha S, Marta C Nunes, William K Rashbaum, Theodore H Schwartz, Robert A Goodman, Guy McKhann, Neeta S Roy, and Steven A Goldman. 2004. 'Fetal and Adult Human Oligodendrocyte Progenitor Cell Isolates Myelinate the Congenitally Dysmyelinated Brain'. *Nature Medicine* 10 (1): 93–97. <https://doi.org/10.1038/nm974>.
- Windrem, Martha S., Neeta S. Roy, Jeremy Wang, Marta Nunes, Abdellatif Benraiss, Robert Goodman, Guy M. McKhann, and Steven A. Goldman. 2002. 'Progenitor Cells Derived from the Adult Human Subcortical White Matter Disperse and Differentiate as Oligodendrocytes within Demyelinated Lesions of the Rat Brain'. *Journal of Neuroscience Research* 69 (6): 966–75. <https://doi.org/10.1002/jnr.10397>.

