

**The effects of hepatitis B vaccination at birth on all-cause early-life morbidity and mortality:
An investigator-initiated randomized controlled trial in Guinea-Bissau**

HBV-NSE (Hepatitis B vaccination and non-specific effects) Trial Protocol

Version 2.2. August 4, 2026.

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STATEMENT OF COMPLIANCE

This document is a protocol for a randomized clinical research study. The study will be conducted in compliance with all stipulations of this protocol and the conditions of ethics committee approval.

The protocol was approved by the Guinea-Bissau Committee of Ethics (CNEPS) on November 5, 2025, approval number **036-CNES-INASA-2025**.

The revised protocol was approved by CNEPS on July 14, 2026, approval number **06-CNEPS-INASA-2026**. The protocol was also submitted to the Research Ethics Committee of the University of Southern Denmark (SDU REC). On 30 September 2026, SDU REC issued a positive advisory assessment (Case no. **26/55358 / REC762**). Pursuant to the decision of SDU's Executive Board of 16 April 2026, SDU REC assessments are advisory. The formal approval to proceed with the revised hepatitis B study was taken by the Dean of the Faculty of Health Sciences on October 7, 2026.

The present revised version includes maternal screening for Hepatitis B Surface Antigen (HBsAg, indicative of active hepatitis B infection), maternal screening for HIV-1/HIV-2, and the use of a placebo.

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PROTOCOL SYNOPSIS

Title: *The effects of hepatitis B vaccination at birth on all-cause early-life morbidity and mortality: An investigator-initiated randomized controlled trial in Guinea-Bissau*

Objective: To determine whether a single dose of monovalent hepatitis B (HBV0) provided as soon as possible after birth and within the first week of life (<8 days), is associated with the composite outcome of all-cause mortality and non-fatal hospital admission with infection within the first 6 weeks of life (before other vaccines are administered), with atopic dermatitis (AD) by 2 years of age and neurodevelopment by 5 years of age, when compared with placebo (control).

Both randomization groups will receive the recommended neonatal BCG and OPV0 vaccines, co-administered with HBV0 in the intervention group. The control group will receive placebo (sterile saline). Prior to enrolment and provided that separate informed consent is provided, the mother will be screened for hepatitis B infection using a HBsAg rapid diagnostic test and offered HIV-1/HIV-2 rapid diagnostic testing. Newborns born to HBsAg-positive mothers will be excluded from the trial and offered HBV0 immediately to reduce the risk of vertical transmission of hepatitis B virus.

GLOSSARY OF ABBREVIATIONS

ABBREVIATION TERM

AD, Atopic Dermatitis
ART, Antiretroviral Therapy
aOR, Adjusted Odds Ratio
aRR, Adjusted Risk Ratio
BCG, Bacille Calmette-Guérin
BHP, Bandim Health Project
CI, Confidence Interval
Co-PI, Co-Principal Investigator
CRF, Case Report Form
DSMB, Data and Safety Monitoring Board
DTP, Diphtheria-Tetanus-Pertussis

HepB, Hepatitis B vaccine in infancy (part of the pentavalent vaccine)

HBeAg, Hepatitis B e antigen

HBsAg, Hepatitis B surface antigen

HBV0, Hepatitis B vaccine birth dose

HDSS, Health and Demographic Surveillance System

Hib, Hemophilus influenzae type b

HIV, Human Immunodeficiency Virus

HNSM, Hospital Nacional Simão Mendes

HR, Hazard Ratio

IPV, Inactivated Polio Vaccine

LMICs, Low- and Middle-Income Countries

MD, Medical Doctor

NSEs, non-specific effects (of vaccines)

PCV, Pneumococcal Conjugate Vaccine

PI, Principal Investigator

PS, Procurement Services (UNICEF)

OPV0, Oral Polio Vaccine birth dose

RCT, Randomized Controlled Trial

RDT, Rapid Diagnostic Test

ROTA, Rotavirus vaccine

SAP, Statistical Analysis Plan

SDU, University of Southern Denmark

SSA, Sub-Saharan Africa

1. INVESTIGATORS AND FACILITIES

1.1 Trial Locations

Trial coordination, data entry, storage of trial materials:

Bandim Health Project (BHP), Bandim, Bissau, Guinea-Bissau.

Trial recruitment sites

Initial recruitment site:

Hospital Nacional Simão Mendes, Bissau, Guinea-Bissau.

Additional recruitment sites (pending approval):

Bandim Health Center, Bandim, Bissau, Guinea-Bissau.

Cumura Hospital, Cumura, Guinea-Bissau

Hospital Militar, Bissau, Guinea-Bissau

1.2 Study Management

1.2.1 Principal Investigator

The Principal Investigator (PI) for this trial is Frederik Scholtz-Buchholzer, MD, PhD, Assistant Professor, Bandim Health Project, University of Southern Denmark.

Co-PI is Professor Christine Stabell Benn, MD, PhD, DMSc, Bandim Health Project, University of Southern Denmark.

1.2.2 Statistician

The senior trial statistician will be Sebastian Nielsen, PhD, Assistant Professor, University of Southern Denmark.

1.2.3 Independent Data and Safety Monitoring Board

An independent Data and Safety Monitoring Committee (DSMB) will be established by the study group prior to the initiation of the trial. The DSMB's Terms of Reference will be established at the inception of the trial, outlining the Board's purpose, composition, responsibilities, and operational procedures. The DSMB will consist of a minimum of three senior clinicians or researchers, including at least one statistician. Trial progress will be monitored through 6-monthly blinded progress reports submitted to the DSMB by the trial team. The DSMB will review any major proposed modifications to the trial (such as termination, or an increase in sample size) and provide advice to the PI and team. The DSMB will convene face-to-face or by conference call before the start of the trial, after half the proposed number of newborns have been enrolled and followed-up, and again after three-quarters have been enrolled - or as determined by the DSMB. The DSMB will review the trial design, informed consent process, data collection forms, safety measures, the statistical analysis plan, and visit the trial site once during the conduct of the trial. Predefined stopping guidelines for safety and efficacy, including interim monitoring procedures, will be specified in the DSMB charter.

1.2.4 Trial Consortium

Assistant Professor **Frederik Scholtz-Buchholzer**, dept. of Clinical Research, SDU has 14 years of experience with the conduct of trials at the Maternity Ward[1–6] of Hospital Nacional Simão Mendes in Bissau, Guinea-Bissau.

The Co-PI, Professor **Christine Stabell Benn**, dept. of Clinical Research, SDU is a co-discoverer of non-specific effects (NSEs) of vaccines and has three decades of experience with the conduct of RCTs in Guinea-Bissau, with established expertise in the bridging of vaccine epidemiology and immunology.

The PI will lead the planning, conduct and reporting of the trial, in close collaboration with the Co-PI, who was the PI's PhD supervisor. The two have had a strong long-standing research collaboration since 2012.

Assistant Professor **Sebastian Nielsen**, dept. of Clinical Research, SDU, equally has long-term experience from the setting, including in the conduct and reporting of RCTs[7–9], and will be the senior trial statistician.

MD, PhD **Cesário Martins**, National Institute of Public Health (INASA), Guinea-Bissau, has worked with the Bandim Health Project for several decades and has extensive experience from the setting.

MD, PhD Fellow **Isaquel Silva**, dept. of Clinical Research, SDU, has worked with the BHP for several years and has experience from the conduct of clinical trials[9–11] in the setting.

Professor **Peter Aaby**, Bandim Health Project, established BHP 48 years ago, discovered NSEs of vaccines and has invaluable experience from conducting RCTs in the setting.

1.3 Sponsor

The Sponsor of the trial is the University of Southern Denmark.

1.4 Funding and Resources

External funding to finance the trial is necessary to fund the activities and has been granted. By December 2025, financial support for conducting the trial has been awarded from Pershing Square Foundation, Bluebell Foundation, and the US CDC. The external funders have no role in the planning of the trial and will have no role in the conduct or reporting of the trial.

2. INTRODUCTION AND BACKGROUND

2.1 Background Information

WHO recommends three vaccines at birth in low-income settings: Bacille Calmette Guérin (BCG) vaccine against tuberculosis, oral polio vaccine (OPV0) and hepatitis B vaccine (HBV0). While much is known about the overall health benefits of BCG and OPV0 at birth, less is known about HBV0. The present study therefore focuses on investigating the overall health effects of HBV0 at birth.

Non-specific effects of vaccines. At the Bandim Health Project (BHP), a long-standing research collaboration between Guinea-Bissau's Ministry of Health and the University of Southern Denmark (SDU), 48 years of population data has uncovered that vaccines can have **non-specific effects (NSEs)**, also at times referred to as *off-target*, *heterologous*, *pathogen-agnostic* or *secondary vaccine effects*, i.e. beneficial or detrimental effects not explained by the specific protection against the targeted disease.[12,13] NSEs are at least partially mediated by induction of *trained immunity* resulting in a modified innate immune response to subsequent unrelated infections.[12,14] Recent experimental work has further demonstrated how vaccination strategies can induce broad protection against heterologous respiratory pathogens through mechanisms involving trained innate immunity and tissue-resident memory responses. For example, mucosal vaccination in mice with toll-like receptor agonists was shown to confer broad, durable protection from diverse respiratory threats, supporting the concept of "universal vaccines" inducing immunity that extend beyond pathogen-specific effects.[15]

In a pre-specified meta-analysis of three randomized controlled trials (RCTs) in Guinea-Bissau, administration of BCG-Denmark given soon after birth reduced all-cause neonatal mortality in low-birth-weight newborns by 38% (17%-54%), with infections and most notably neonatal sepsis accounting for the excess deaths among controls.[1,2] In addition, there tended to be fewer hospital admissions with sepsis by 6 weeks of age among neonates randomized to BCG at birth.[2] The beneficial effect of BCG-Denmark lasted until a different vaccine was given at 6 weeks of age. Providing OPV0 may further reduce mortality when given with BCG during the first two days of life,

the mortality reduction in a trial in Bissau that compared BCG+OPV0 vs BCG alone being 42% (10%-62%).[16] This effect was especially evident for males and OPV0 was especially efficacious against fatal respiratory infections.[17] The findings regarding BCG led to national vaccine policy changes in Guinea-Bissau and inspired related research globally.[4,18–25]

In a recent cluster-RCT from Guinea-Bissau, providing BCG + OPV0 at home visits within 72 hours of birth was associated with a 59% (8%-82%) reduction in early infant mortality, when compared with home visits without provision of vaccines.[24] A recent RCT from India in babies weighing below 2,000 g reproduced the finding of significantly fewer cases of fatal neonatal sepsis and reduced all-cause mortality risk when BCG + OPV0 was provided shortly after birth, compared with vaccination at discharge.[25] The findings included a marked reduction in the risk of fatal neonatal sepsis caused by *Klebsiella Pneumoniae*, a leading cause of neonatal sepsis in low- and middle-income settings.[25]

Hepatitis B. Hepatitis B virus is a major global health concern, not least in sub-Saharan Africa (SSA), where it is a leading cause of chronic hepatitis, cirrhosis and fatal liver cancer.[26–28] Infection acquired perinatally from mother to child (vertical transmission) or during early childhood (horizontal transmission) carries an elevated risk of chronic infection and severe disease downstream.[28] The risk of vertical transmission of hepatitis B is highest (70-90%) in mothers that are both Hepatitis B surface antigen (HBsAg) and Hepatitis B e antigen (HBeAg)-positive, and 10-40% among mothers that are HBsAg-positive and HBeAg-negative.[29–31] In SSA, approximately 10–25% of HBsAg-positive pregnant women are HBeAg-positive, and the overall risk of mother-to-child transmission without prophylaxis is estimated to be 10–15%.[32,33]

Hepatitis B epidemiology in Guinea-Bissau

In a small cross-sectional study utilizing blood samples from children aged 18 months, who were born before HBV vaccination was introduced in Guinea-Bissau in 2008 as part of the pentavalent vaccine series, 11% (21/187) were infected with Hepatitis B (HBsAg-positive) and 5% had recovered from infection.[34] A population-based study conducted by BHP between 2014-2016 reported a high (18.7%, 15% in women, 24% in men) prevalence of HBsAg among adults in urban Bissau.[35] In more recent (2022) modelled estimates from WHO, the prevalence of HBsAg-positivity among children <5 years of age is estimated to be 2%[36] and 12% among adults.[37] Vertical transmission of hepatitis B is thus a relevant public health concern in Guinea-Bissau, which could be addressed by HBV0 vaccination.

Hepatitis B vaccination. HBV0 is the most effective approach to prevent vertical transmission[28], with the subsequent pentavalent vaccines provided at 6, 10, and 14 weeks of age, introduced in 2008[38], providing protection against horizontal hepatitis B transmission.[39]

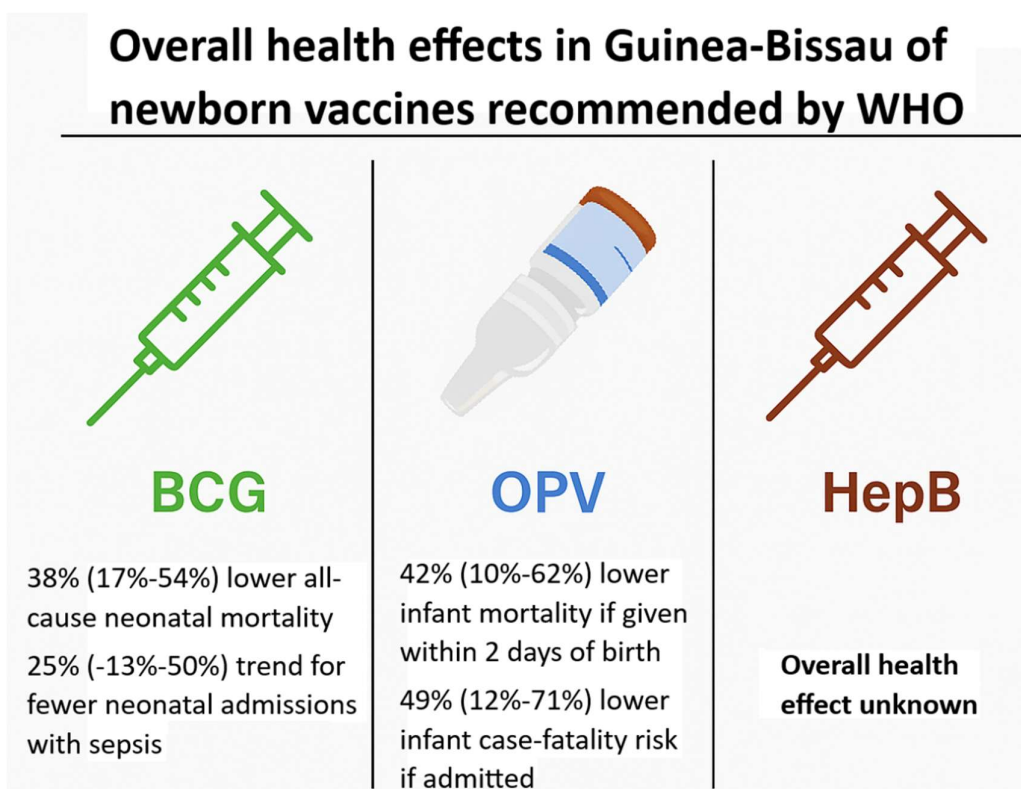
A systematic Cochrane review of RCTs has shown that HBV0 reduces vertical transmission by 72% (60%-80%), but noted limited data on adverse events.[40] The use of HBV0 is especially important in countries with a high HBsAg prevalence, yet WHO recommends universal HBV0 (including in neonates with a birth weight <2,000g and premature neonates), also in high-income countries.[28] While Australia, New Zealand and Japan have a universal HBV0 policy, most European countries only provide targeted HBV0 to babies born to HBsAg-positive mothers.[41] In the US, the policy was recently updated so that HBV0 is no longer recommended to newborns born to a HBsAg-negative mother.[42] Only 15 of 47 countries in the WHO Africa region have implemented HBV0 due to logistical and contextual barriers and the limited direct benefit when the mother is HBsAg-negative.[39,43] Notably, since most African countries including Guinea-Bissau have no hepatitis B screening program, HBV0 would be given to all neonates, even though it is only needed by those

whose mothers are hepatitis B carriers. Most children are well protected against horizontal transmission by the three HepB vaccines included in the pentavalent vaccines provided at 6, 10 and 14 weeks of age. This makes it particularly important to ensure that there are no negative effects of HBV0. The Ministry of Health in Guinea-Bissau may introduce universal HBV0 for all newborns in 2028, in addition to the currently recommended BCG and OPV0. There is therefore a **window of opportunity to conduct a large-scale RCT evaluating the overall health effects of HBV0** before vaccination may become national policy. The proposed trial will address the significant knowledge gap regarding the overall health effects of HBV0. In addition, the trial incorporates maternal screening for HBsAg with targeted provision of HBV0 to newborns at risk of vertical transmission, as well as maternal HIV-1/HIV-2 testing with counselling and referral. Together, these measures strengthen early-life preventive care and linkage to treatment services in the study setting.

HBV0 and non-specific effects

Hepatitis B vaccine was developed in 1982[28]. It has never been investigated whether HBV0 is associated with NSEs - beneficial or detrimental (Figure 1).

Figure 1. Effects on overall health in Guinea-Bissau of at-birth vaccines recommended by WHO.



The hepatitis B vaccine was developed and introduced at a time when vaccine evaluation focused primarily on pathogen-specific protection and safety, preceding the discovery of NSEs. Accumulating epidemiological, immunological and experimental advances have since demonstrated that vaccines may influence susceptibility to unrelated diseases, and that current frameworks for testing and evaluating vaccines does not fully capture such broader effects.[44] This evolving understanding has led to calls for a broader conceptual framework in vaccinology that considers overall health effects, interactions between vaccines, and context-dependent responses.[12] Following systematic reviews of the evidence on non-specific effects of vaccines,

WHO's Strategic Advisory Group of Experts on Immunization (SAGE) concluded that the available data were insufficient to warrant changes in vaccination policy, but emphasized the need for further high-quality research, specifically encouraging the conduct of randomized controlled trials to provide definitive evidence.[45,46]

In a randomized trial conducted in The Gambia and Papua New Guinea, the immunogenicity of hepatitis B vaccination varied depending on whether it was administered alone, co-administered with BCG, or provided after BCG, indicating that neonatal immune responses are highly sensitive to early-life vaccine exposures and their combinations.[47] Two observational studies from West Africa have suggested that HepB vaccines provided later in infancy may be associated with increased female mortality (NSEs).[48,49] Increased female mortality has also been seen after other non-live vaccines.[48,49] There is thus a critical evidence gap for an effective and universally recommended intervention.

HBV0 and atopic dermatitis

Several studies have found that delaying the first dose of aluminum-adjuvanted pentavalent vaccine is associated with a reduced risk of developing AD. In Australia, diphtheria-tetanus-pertussis (DTP) aluminum-adjuvanted vaccine is recommended at 2 months of age. In a study of 4,433 Australian children, receiving DTP with one month's delay was associated with reduced risk of eczema, aOR: 0.57 (0.34-0.97) and reduced use of eczema medication, aOR: 0.45 (0.24-0.83).[50] In a subsequent study of 143,429 Danish children, for whom DTP is recommended at 3 months of age, one month's delay was also associated with a reduced risk of eczema, aRR 0.94 (0.91-0.97), which was further reduced if the second dose was delayed as well, aRR=0.88 (0.82-0.93).[51]

In a recently published Danish study[52], it can be deduced that the incidence rate of AD among children who did not receive aluminum-containing vaccines was 0.6% (229/36,678), vs 0.9% (22,749/2,666,948) for those that had received an aluminum-containing vaccine. While not adjusted for confounders, this provides a crude relative risk of 0.73 (0.64-0.83) for those who did not get aluminum-containing vaccines vs those who received aluminum-containing vaccines. Similar analyses point in the same direction for the combined group of atopic and allergic outcomes, the relative risk being 0.81 (0.75-0.89) ([Rapid response](#) to [52]).

HBV0 and neurodevelopment

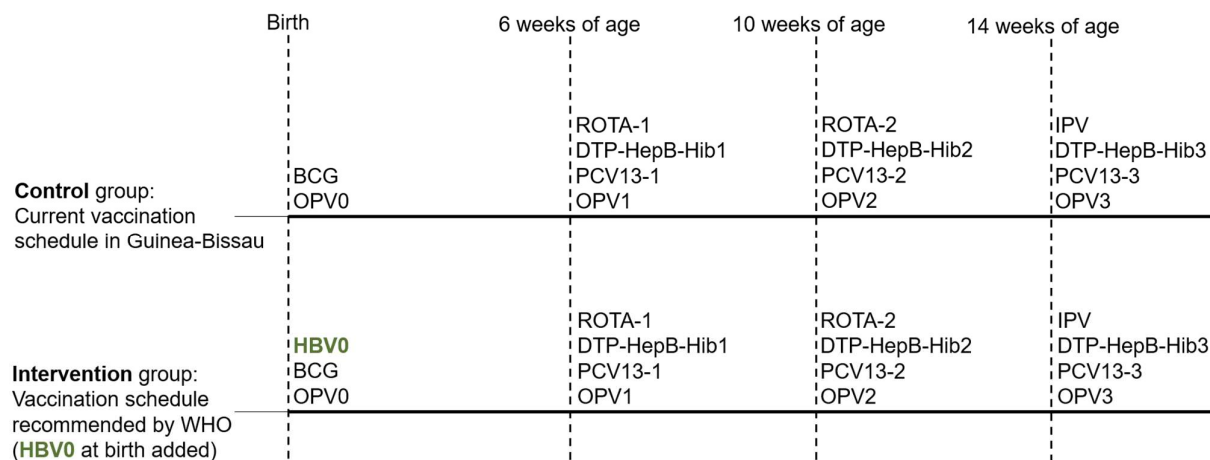
Aluminum adjuvants are widely used in vaccines and have been extensively studied; however, concerns have been raised regarding providing an aluminum-containing vaccine within 24 hours after birth[53], as aluminum has known neurotoxic properties at high exposure levels.[54–56] This is particularly relevant in infancy[57] and aluminum adjuvants have been speculated to be associated with neurodevelopmental disorders.[58,59] The recent Danish study[52] mentioned above found no association between the cumulative dose of aluminum received by age 2 years and subsequent development of neurodevelopmental disorders in 1,224,176 children, but Denmark does not use at-birth vaccines; the first dose of aluminum-containing pentavalent vaccine is provided at 3 months of age, so the study does not exclude an association between at-birth aluminum exposure and the risk of downstream neurodevelopmental disorders. This research question has not been evaluated in randomized trials.

2.2 Rationale for Current Study

The available evidence suggests that BCG and likely also OPV0, especially given in combination, reduces all-cause neonatal mortality due to fewer cases of fatal sepsis and fewer respiratory infections.[1,2,4,16,18,24,25] Adding monovalent Hepatitis B vaccine would address the relevant public health threat of vertical Hepatitis B virus transmission, which occurs in 10%-15%[33] of

children born to the ~12%[37] of mothers in Guinea-Bissau that are HBsAg-positive, e.g., in ~1.5% of all newborns. Universal HBV0 could decrease this risk to approximately ~0.4%, an absolute risk reduction of vertical hepatitis B transmission of ~1.1%, but the effect on overall health is unknown. Because up to 90% of perinatally infected infants develop chronic infection, and 15–25% of adults with chronic infection die prematurely from cirrhosis or liver cancer[60], the long-term burden of disease attributable to perinatal infection remains substantial. These benefits must, however, be weighed against the possibility that HBV0 could have broader effects on early-life health outcomes, including possible effects on susceptibility to unrelated infections, allergic disease, or neurodevelopmental outcomes. A randomized trial that compares the current neonatal vaccination schedule in Guinea-Bissau, BCG + OPV0, with the WHO-recommended, future schedule of BCG + OPV0 + HBV0 (**Figure 2**), is therefore needed to assess whether HBV0 co-administered with BCG + OPV0 provided to healthy newborns born to HBsAg-negative mothers modulates the risk of these outcomes, which needs to be counterbalanced against the known benefits of HBV0.

Figure 2. The current vaccination schedule in Guinea-Bissau from birth to 14 weeks of age (control group, top) and the WHO-recommended vaccine schedule (intervention, bottom)



Abbreviations: BCG, Bacille Calmette-Guérin; OPV0, Oral Polio Vaccine; HBV0, Hepatitis B vaccine at birth; HepB, Hepatitis B vaccine in infancy; ROTA, Rotavirus vaccine; DTP, Diphtheria-Tetanus-Pertussis; Hib, Hemophilus Influenzae; PCV, Pneumococcus vaccine; IPV, Inactivated Polio Vaccine.

There are several different possible strategies to prevent transmission of hepatitis B employed in SSA (**Table 1**).

Table 1. Three strategies to prevent vertical transmission of hepatitis B in SSA

Vaccination strategy	Description
Routine infant pentavalent vaccination from 6 weeks of age, employed by ~27 countries in the WHO Afro Region[61]	HepB vaccine is given as part of the routine pentavalent schedule (e.g., at 6, 10 and 14 weeks of age) without a birth dose, mainly preventing horizontal transmission in infancy (Current policy in Guinea-Bissau).
Universal HepB birth dose vaccination + pentavalent vaccination series, employed by ~18 countries in the WHO Afro Region[61]	All newborns should receive a hepatitis B vaccine dose up to 7 days after birth but ideally within 24 hours of birth, regardless of maternal HBsAg status, in addition to the three doses in the routine pentavalent schedule (Future policy in Guinea-Bissau).
Maternal screening with targeted birth dose vaccination, employed by 2 countries (Mauritius, South Africa) in the WHO Afro Region[61]	Pregnant women are tested for HBsAg during antenatal care, and only newborns born to infected mothers receive HBV0 (with additional prophylaxis if available).

In case HBV0 has positive or no non-specific effects, a universal vaccination strategy would be the most feasible and logistically simple approach in most settings and if HBV0 is associated with detrimental non-specific effects, a targeted vaccination strategy may be more appropriate. The trial will therefore provide evidence to inform whether universal or targeted HBV0 strategies are most appropriate in Guinea-Bissau and similar settings.

2.3 Research Questions

Does the administration of a single dose of neonatal monovalent HBV0 to newborns within 1 week of age reduce or increase all-cause mortality or non-fatal admission with infection (composite outcome) in the first 42 days of life?

Are there sex-differential non-specific effects of HBV0?

Does the administration of a single dose of neonatal monovalent HBV0 to newborns within 1 week of age affect the risk of AD by 2 years of age?

Does the administration of a single dose of neonatal monovalent HBV0 to newborns within 1 week of age affect neurocognitive outcomes by 5 years of age?

3. STUDY OBJECTIVES

3.1 Primary Objective

We will investigate whether the administration of a single vaccine dose of pediatric monovalent HBV0, co-administered with BCG and OPV as soon as possible after birth within the 1st week (<8 days) of life, affects the risk of the composite outcome of all-cause mortality and non-fatal hospital admission with infection within 42 days after birth (i.e., before the first routine infant vaccinations scheduled at 6 weeks of age).

3.2 Secondary Objectives

To investigate the effect of HBV0 on AD by 2 years of age.

To investigate the effect of HBV0 on neurodevelopment by 5 years of age.

For all outcomes, it will be assessed whether there are sex-differential effects of HBV0.

4. STUDY DESIGN

4.1 Type of Study

The study will be a single-blind, placebo-controlled, multi-center, phase IV randomized controlled trial enrolling healthy neonates that are ≤ 1 week of age and born to HBsAg-negative mothers. The main recruitment center will be the Maternity Ward of Hospital Nacional Simão Mendes (HNSM), which has approximately 8,000 births/year. The HNSM Director approved to host the trial on September 12, 2025. The trial will begin at HNSM, and then expand to additional recruitment units, pending approval from each health facility:

Additional recruitment sites (pending approval):

Bandim Health Center (~200 births per year).

Hospital de Cumura (~700 births per year).

Hospital Militar (~1,000 births per year).

All inclusion sites are in the greater Bissau area of Guinea-Bissau. We aim to recruit at least 14,500 children (see sample size calculation) during a 2-year period. Eligible newborns will be randomized 1:1 to receive a neonatal dose of monovalent HBV0 co-administered with BCG + OPV0 vs BCG + OPV0 with placebo (saline).

4.1.1 Eligibility Screening and Participant Recruitment

Eligibility screening will occur at the birth facility, or at the health facility when a newborn born elsewhere comes for vaccination. Maternal screening for HBsAg and HIV-1/HIV-2 will be offered to all women presenting at participating facilities in the peripartum period, irrespective of whether the newborn is eligible for enrolment. If the mother is known to be HBsAg-positive or tests positive for HBsAg at a peripartum rapid diagnostic test (RDT) performed by our team provided separate informed consent, the newborn will be excluded and offered HBV0. In such cases, our team will provide counselling to the mother, with explanation of the importance of HBV0 for future offspring. Our team will offer vaccination with BCG + OPV0 + HBV0 to newborns born to mothers who do not provide informed consent for either the HBsAg RDT or participation in the trial. HBV0 will be provided if the newborn is <8 days of age.

Maternal HIV-1/HIV-2 testing will be offered with separate informed consent. A positive screening test will not affect eligibility, as maternal HIV infection alone does not preclude routine neonatal vaccination at birth. Mothers with a positive screening test will receive confidential counselling and referral for confirmatory testing and linkage to HIV care services in accordance with national guidelines.

Newborns are eligible to participate in the trial if they are clinically stable, unvaccinated, born to a HBsAg-negative mother and <8 days of age. Moribund neonates and children with major malformations will be excluded. Informed written consent to participate in the trial will be obtained from mothers/guardians following an oral explanation in Portuguese Creole and a written trial explanation in Portuguese. Participation is voluntary, and families may withdraw from the trial at any time. Baseline information such as maternal and neonate mid-upper-arm circumference, maternal BCG scar status, socio-economic factors and phone contacts for the mother, the father and family members will be collected, as done previously.[3,5,6]

Randomization and blinding: Randomization will be performed using sealed envelopes prepared in blocks of 24, stratified by sex and place of inclusion. After the informed consent procedure, the mother/guardian selects a sealed envelope allocating the neonate to either the intervention group (HBV0) or the control group. While the inclusion team will not be blinded to group allocation, the mother/guardian and all outcome assessors involved in follow-up procedures and the trial statistician will be blinded, ensuring objective outcome assessment while maintaining operational feasibility.

Intervention: All enrolled neonates will receive BCG (0.05 mL intradermal injection in the left deltoid) and OPV0 (2 oral drops of bivalent OPV), in accordance with national guidelines. The intervention group will additionally receive 0.5 mL of monovalent HBV0 intramuscularly in the anterolateral aspect of the thigh, while the control group receives placebo (sterile saline solution), consisting of 0.5 mL sterile saline, also administered intramuscularly in the anterolateral thigh.

At HNSM, BCG and OPV0 are currently provided to newborns only on weekdays, with there at times being situations with national vaccine stockout, although these are rare. At the smaller hospitals and health centers, OPV0 is provided daily on weekdays while BCG is typically available on one vaccination day during the week, to reduce vaccine wastage. There are currently nationwide restrictions on the opening of BCG vials, with a rule that 15 eligible infants must be present before a vial is opened, to reduce vial wastage. The trial team will ensure that the necessary vaccines (BCG,

OPV0, HBV0) are available on all days of the week (including holidays and weekends) at HNSM and on normal workdays (excluding holidays and weekends) at the smaller inclusion sites, and that vaccine vials for all three vaccines are opened as soon as one eligible newborn is present.

4.2 Follow-up Measures

To ensure comprehensive outcome data, follow-up of all children will be conducted through 5 complementary mechanisms, including telephone interviews (A), home visits (B), hospital surveillance (C), investigation of AD incidence up to 2 years of age (A, B, D), and a neurodevelopmental assessment by 5 years of age (E).

A) Telephone interviews at age 1 week, 6 weeks, 6 months, 1 year and 2 years of age.

Families will be contacted to assess vital status, hospital admissions, adherence to the recommended routine vaccination schedule (at 6, 10 and 14 weeks), and morbidity. Separate informed consent will be obtained for follow-up contact related to HIV care and linkage to services. For mothers with a positive HIV screening test who have provided consent for follow-up contact regarding test results, a brief additional check will be conducted to ensure successful linkage to care and initiation of recommended maternal and neonatal management. If an infant has died, brief information will be collected regarding the circumstances such as date, location (home or hospital), and symptoms. In prior BHP trials, 99% of families provided at least 1 telephone contact, and >95% successfully provided data on vital status by 6 weeks of age (Table 2).[5]

Table 2. BCGSTRAIN II[5] Telephone follow-up data.

Mother supplied telephone number(s) at inclusion	99%
Follow-up success rate (combined for 6-week and 6-month calls)	95%
Successful 6-week follow-up if two phone numbers provided	88%
Successful 6-week follow-up with ≥three phone numbers provided	97%

This demonstrates the feasibility and minimal loss to follow-up with telephone-based interviews in our setting. Each reported death will be sought separately confirmed by two different follow-up sources (e.g., two separate telephone contacts or one telephone contact and hospital admission outcome data or BHP Health and Demographic Surveillance System (HDSS) study area data).

B) Cohort of BHP HDSS study area infants: home visit by 2 months and 12 months of age

Approximately 15% (~2,100 with 14,500 inclusions) of the newborns will reside in BHP's HDSS study area. These infants will receive a home visit at 2 and 12 months of age to assess vital status, BCG scar status and morbidity incidence, as done in previous trials.[1,3–5] In addition, the families will be asked about previous symptoms of AD and the infant will be examined for signs of AD. In case AD is suspected, the child will be referred to an AD specialist (see below). For HDSS infants that die, a verbal autopsy will be performed 3-6 months after the death.

C) Hospital admission surveillance

All admissions to the national hospital's pediatric ward HNSM are monitored and entered daily by a dedicated BHP team.[2,3,62,63] Parental names and telephone numbers are registered for all admissions and will be used for data linkage and confirmation. Admission charts linked via a unique trial participant vaccination card sticker ID include the diagnosis and admission outcome. For approximately 60% of admitted infants, data from the vaccination card including the unique ID is documented during the admission. To identify remaining admissions not registered with the unique trial ID, a previously validated standardized probabilistic record matching protocol will be applied.[2,3,5] All admissions will be manually vetted by a blinded senior researcher. To assess the likely cause of death or admission, we will use both hospital data, telephone follow-up data, and verbal autopsy data from the BHP study area.

D) Assessment of AD

At the telephone contacts and HDSS home visits, we will ask the mother whether the child has an itchy rash. If the caregiver reports that the child has scratched repeatedly and/or experienced sleep disturbances due to itching and the family resides in the greater metropolitan area of Bissau, a home visit will be offered by a physician experienced in diagnosing AD at a time point where the child suffers from the rash.

At the home visit, the physician will perform a clinical examination to assess whether the child has AD or another condition. Appropriate treatment will be initiated, and guidance will be given for clinical follow-up. At follow-up visits, it will be ascertained if the treatment has helped, and/or if further/other treatment is needed. The data on 1) self-reported AD symptoms and 2) physician-diagnosed AD will be reported separately for the AD outcome.

E) Neurodevelopmental assessment

A subgroup of 500 singleton BHP HDSS children will receive a neurodevelopmental assessment at 5 years of age, provided renewed informed consent. The evaluation will take place at the local health center.

4.3 Outcome Measures

Primary outcome: Mortality and morbidity

The composite outcome of all-cause mortality or first non-fatal hospital admission with infection from randomization to 42 days of age (before the next routine vaccination) in the group given HBV0 compared with the control group. Infectious admissions are defined based on the primary discharge diagnosis recorded at the hospital, classified according to predefined diagnostic categories (see appendix section 14.2 for details), predefined before trial unblinding in the Statistical Analysis Plan.

Secondary outcomes: Neurodevelopmental disorders and AD

AD by 2 years of age

Cumulative incidence of AD symptoms up to 2 years of age, based on caregiver-reported symptoms collected during telephone follow-up and home visits, and cumulative incidence of physician-diagnosed AD in a subset of children (see sample size calculation) with suspected AD who are offered clinical assessment by a physician with special training in AD diagnosis. The two outcomes will be reported separately.

Neurodevelopment by 5 years of age

In a subgroup of 500 singleton children from the HDSS followed to 5 years of age: cognitive outcomes assessed by the Mullen Scales of Early Learning and the Kaufman Assessment Battery for Children. These pediatric tests have been tested for validity in Sub-Saharan Africa.[64–67] Outcomes will be further explored to assess effects by subcategory of outcome and age (**Table 3**).

Table 3. Trial outcomes and data sources

Primary outcome	Population	Age assessed	Data source(s)
Composite outcome: all-cause mortality and first non-fatal hospital admission with infection	All infants	6 weeks	A, B, C
Secondary outcomes			
I: Self-reported AD and II: physician-diagnosed AD	I: All infants. II: To 200 cases among controls*	2 years	A, B, D
Neurodevelopmental outcomes	500 HDSS children*	5 years	E
Outcome subcategories and by age			
Composite outcome: all-cause mortality and admission due to non-fatal infection	All infants	2 years	A, B, C
All-cause mortality	All infants	6 weeks, 2 years	A, B, C

In-hospital case-fatality (all-cause, infectious diseases)	Hospitalized infants	6 weeks, 2 years	C
Hospital admission for major diseases groups (neonatal conditions aside from sepsis, respiratory infections, bronchitis, diarrheal diseases, malaria, malnutrition-related diseases)	All infants	6 weeks, 2 years	C

* See sample size calculation section below for further information.

4.4 Sample Size

Based on a recently completed trial of BCG-Denmark+OPV0 vs BCG-Bulgaria+OPV0[6], conducted with the proposed setup and follow-up methodology, we anticipate a 6-week mortality rate of 1.4% and a non-fatal infectious disease admission rate of 2.6%, for a composite risk of 4% in the control group. The number of dropouts in previous trials has been low, and the composite risk of 4% is based on the observed data in the most recent trial[6], e.g., the rate accounts for loss to follow-up. If the rate of the composite outcome of all-cause mortality or non-fatal hospital admission with infection is 4% (0.04) in the control group, and HBV0 increases the rate of the composite outcome by 25% (to $1.25 \times 0.04 = 0.05$) or decreases the rate by 22% (to $0.78 \times 0.04 = 0.0312$), to have 80% power to detect such changes for the primary outcome, with alpha error <0.05 , the required sample size is 14,368 newborns, 7,184 in each group, from sampsi .050 .040, a(0.05) p(0.80) and sampsi .040 .0312, a(0.05) p(0.80) in Stata/BE version 18.0. For simplicity, enrolling 14,500 newborns is expected to be possible before HBV0 is introduced in Guinea-Bissau. The trial is powered for 14,500 participants; however, enrolment may continue up to a maximum of 25,000 participants to increase statistical precision, unless HBV0 is introduced nationally before this threshold is reached. With a composite outcome prevalence of 4% (0.04), the number of deaths and non-fatal infectious disease admission expected is $7,250 \times 0.04 = 290$. With 290 events in the control group, expected to be reached when 7,250 newborns have been recruited in each trial arm, the trial has 80% power to detect if HBV0 has a beneficial non-specific effect, decreasing the rate of the primary outcome by $\geq 22\%$ or a detrimental non-specific effect, increasing the rate by $\geq 24\%$. This design ensures adequate statistical power to detect clinically meaningful differences in the primary outcome and allows for a reasonably precise estimation of the potential non-specific effects of HBV0. The exact timepoint that the trial will end is determined by the time when the Ministry of Health introduces HBV0 in Guinea-Bissau. We will therefore continue the trial until HBV0 is introduced in Guinea-Bissau or when 25,000 neonates have been enrolled in the trial, whichever comes first. If 25,000 neonates are enrolled in the trial, we will be able to detect an increase in the rate of the composite outcome of 18% and a decrease of 17%.

For AD, little is known about the prevalence in Sub-Saharan Africa, but the available data indicate prevalence rates similar to European Union countries, e.g., approx. 12% among 5-year old children.[68] If the cumulative incidence by 2 years of age is 10%, there would be 725 cases of AD in the control group, if we recruit 7,250 children in each arm of the trial. We aim to conduct home visits to identify cases of AD until 200 cases have been diagnosed among controls, which would provide power to detect a 28% increase (to 256 intervention group cases) in the risk of AD for children who receive HBV0 and a 25% decrease (to 150 intervention group cases).

For cognitive outcomes, a sample size of 500 will make this study a large study with power to detect clinically meaningful differences in cognitive abilities between children who did or did not receive HBV0.[64]

4.5 Expected Trial Duration

We expect to be able to enroll 14,500 newborns within 1.5 to 2 years (Table 4).

Table 4. Trial timeline with 14,500 enrollments

Period	2026	2027	2028	2029	2030	2031
Events	Enrolment of 14,500 children					
Assessment for morbidity and mortality to 6 weeks	Ongoing	Ongoing	Assessment completed			
Assessment for neurodevelopmental outcomes by 5 years of age					Ongoing	Assessment completed
Assessment for AD by 2 years of age	Ongoing	Ongoing	Ongoing	Ongoing	Assessment completed	

Follow-up will be until 2 years of age for mortality and morbidity. Follow-up for the primary outcome will be finished ~2 years after trial initiation (~1½ years of enrolment with primary follow-up assessed at 6 weeks), while secondary follow-up for morbidity, mortality and AD will continue to 2 years of age. The first 500 children recruited from the HDSS will be followed to 5 years of age for neurodevelopmental outcomes. For this reason, the trial data collection should be finished within <7 years.

5 STUDY TREATMENTS

5.1 Treatment Arms

5.1.1 Description

Both groups will be vaccinated at the maternity ward or at the health center where they were recruited, or, in the case of neonates born at home, when they are brought to the health facility for their first vaccinations. All participants will receive BCG and OPV0 plus either HBV0 or saline placebo. Enrolment and vaccination will be administered as soon as possible after birth, ideally within the first 24 hours when feasible.

5.1.2 Dosage and Route of Administration

Intervention: All enrolled neonates will receive BCG (0.05 mL intradermal injection in the left deltoid region) and OPV0 (2 oral drops) at the hospital or health center, in line with national guidelines. OPV0 and BCG will be provided through the national vaccination program to the HNSM inclusion center. For use at the other inclusion centers, and in case of shortage of vaccines, the BHP will procure OPV0 and BCG for the trial via UNICEF's Procurement Services (PS), ensuring that OPV0 and BCG is available for daily vaccination at all trial inclusion sites. BHP has a Memorandum of Understanding with UNICEF. The intervention group will additionally receive a 10 mcg dose (in 0.5 mL suspension) of monovalent HBV0 (Hepatitis B pediatric subunit vaccine, Serum Institute of India (SII))[69] intramuscularly in the anterolateral aspect of the thigh. In case the SII HBV0 vaccine cannot be procured, Euvax B (LG Chem Ltd)[70], BEVAC® (Biological E. Limited)[71] or a similar, WHO-prequalified product will be sourced. Placebo will be sterile saline solution administered intramuscularly in the anterolateral aspect of the thigh.

6 SUBJECT ENROLLMENT AND RANDOMIZATION

6.1 Recruitment

All unvaccinated and healthy neonates born to a HBsAg-negative mother will be eligible for the trial provided that written informed consent is obtained and they fulfil none of the exclusion criteria. Eligibility screening will occur at the recruitment centers, either at the Maternity ward or when neonates are brought there for their first neonatal vaccines. Neonates born at home, presenting at a

recruitment center, are thus eligible if they are less than 1 week old (<8 days of age). Informed written consent will be obtained from mothers/guardians following an oral explanation of the trial in Portuguese Creole and a written explanation in Portuguese. Participation is voluntary, and families may withdraw from the trial at any time. If the family declines to participate or the child is not eligible for any other reason, the inclusion team will offer to vaccinate the child with BCG, OPV0 and HBV0 according to the age of the infant (HBV0 if <8 days of age, OPV0 if <15 days of age (national policy) and BCG if < 90 days of age (national policy)), unless there are known contraindications for providing one or more of the vaccines.

6.2 Maternal Screening for Hepatitis B Surface Antigen (HBsAg) and HIV-1/HIV-2

For the proposed trial, maternal screening for HBsAg will be implemented as part of the eligibility assessment prior to enrolment. Mothers will be offered testing for hepatitis B surface antigen (HBsAg) using a WHO-prequalified RDT (e.g., [72]) performed on a capillary blood sample obtained by finger prick. Testing will be performed prior to the enrolment procedure, provided that separate informed consent for the screening procedure has been obtained. The screening will be performed as soon as possible after the mother's arrival to the maternity ward or recruitment facility to ensure timely identification and vaccination of newborns at risk of vertical hepatitis B transmission. The inclusion team will ensure that the mother's privacy is protected by providing a personal, confidential space where the result of the test can be communicated. Those who test positive for HBsAg will receive post-test counselling from a medical doctor or trained counsellor that is available on site to provide support and answer questions. Counselling will include information on hepatitis B infection, the importance of adhering to the neonatal and pentavalent vaccination series, and recommendations for ensuring that HBV0 is administered to any future offspring. BHP will maintain a stock of HBV0 vaccines for this purpose, and we will inform the mother to inform BHP of future pregnancies until the HBV0 recommendation has been implemented by the Ministry of Health in Guinea-Bissau.

HBsAg-positive mothers represent a heterogeneous group with respect to infectivity. It is mainly a subset - particularly those with high viral load or HBeAg positivity – that are likely to transmit hepatitis B virus to their newborns. Because HBeAg markers are not routinely measured in this setting, all infants known or found to be born to HBsAg-positive mothers will not be randomized but instead offered HBV0 as a precautionary measure. HBV0 will be provided as soon as possible following the positive test result to maximize the protective efficacy and minimize the risk of vertical transmission, in accordance with WHO recommendations to prevent mother-to-child transmission of hepatitis B.

Management of infants when maternal HBsAg screening is declined or unavailable

Newborns whose mothers decline HBsAg testing, or for whom maternal HBsAg status cannot be determined before enrolment, will not be randomized. Because the maternal HBV infection status remains unknown, their newborns will be offered hepatitis B birth-dose vaccination as soon as possible, ideally within 24 hours of birth, together with BCG and OPV0 according to national policy. It will be made clear that refusal of maternal screening or refusal to participate in the trial will not affect access to vaccination, clinical care, or other services.

Management of Infants Born to HBsAg-Positive Mothers

Neonates born to mothers who test positive for HBsAg will not be eligible for randomization and will instead be offered immediate vaccination as soon as possible with BCG, OPV0 and HBV0 as targeted prophylaxis according to the study procedures. Hepatitis B Immune Globulin is not available in Guinea-Bissau and will therefore not be administered.

HIV screening, counselling, and referral

Mothers will also be offered testing for HIV-1/HIV-2 using a WHO-prequalified RDT performed on a capillary blood sample obtained by finger prick, provided separate informed consent is obtained. Testing will be conducted in a confidential setting, and results will be communicated privately to the mother. Mothers with a positive screening test will receive post-test counselling from a medical doctor or trained counsellor. In case of an indeterminate test result, repeat testing will be performed in accordance with national guidelines. Counselling will include information on HIV infection, the availability and effectiveness of antiretroviral therapy, and measures to reduce the risk of mother-to-child transmission. Mothers with a positive screening test will be referred for confirmatory testing and linkage to HIV care services in accordance with national guidelines in Guinea-Bissau. Newborns of mothers with confirmed HIV infection will be referred for initiation of neonatal prophylaxis and follow-up according to national prevention of mother-to-child transmission guidelines. Participation in the trial will not be contingent on acceptance of HIV testing, and maternal HIV status will not constitute an exclusion criterion.

6.3 Eligibility Criteria

6.3.1 Inclusion Criteria

Neonates will be eligible for enrolment if all the following criteria are met:

- The neonate is present at one of the recruitment centers.
- The neonate is clinically stable at the time of enrolment.
- The mother has provided informed consent for HBsAg testing.
- The maternal HBsAg test result is negative based on screening performed prior to enrolment.
- Written informed consent for trial participation has been provided by a parent or legal guardian (mother, father, or guardian).

6.3.2 Exclusion Criteria

Participants fulfilling any of the following will be excluded:

- Age more than 1 week (≥ 8 days of age).
- Already received any vaccine prior to enrolment.
- Is moribund, defined as having a high likelihood of imminent death based on severe clinical condition, as assessed by trained clinical staff.
- Major visible congenital malformation.
- Multiple births (twins) of three or more infants.

6.4 Randomization Procedures

After informed consent has been obtained, enrolment data will be documented on a written Case Report Form (see below). From a collection of sealed randomization envelopes, prepared in blocks of 24 and stratified by sex and place of inclusion, the mother selects a sealed envelope allocating the neonate 1:1 to the intervention group (HBV0), or the control group (saline placebo). To avoid confusion at subsequent follow-up, twins of the same sex are only randomized once and allocated the same treatment (the mother only draws one sealed randomization envelope), whereas twins of different sex will be randomized according to their sex.

6.5 Blinding Arrangements

The trial is designed as a prospective, randomized, single-blind, placebo-controlled design with blinded endpoint adjudication and blinded statistical analysis. The blinding arrangements are structured to minimize bias while ensuring clinical safety and operational feasibility within a high-volume setting.

6.5.1 Blinding of Participants and Guardians

The mother or legal guardian will remain blinded to the treatment assignment throughout the study. The use of a saline placebo injection for the control group injected in the same location as the intervention ensures that the appearance of the intervention is similar across both study arms.

Physical Masking: Monovalent HBV0 is a milky liquid, whereas the saline placebo is clear. To prevent the mother from identifying the substance, the study injection will be prepared at a station away from the mother's direct line of sight. Standard clear syringes will be used unless opaque alternatives can be sourced. In either case, identical volumes (0.5 mL) and syringe types will be used across both arms to ensure the procedure appears identical.

6.5.2 Blinding of Research Assistants, Hospital Staff and Outcome Assessors

- **Inclusion teams:** The personnel responsible for recruitment, screening, and vaccination will not be blinded to the allocation.
- **Follow-up and outcome assessment teams:** Research assistants involved in subsequent follow-up procedures (e.g., home visits, clinical assessments, and hospital record reviews) will have no access to the randomization logs or the vaccination section of the Case Report Forms. All vaccination-related data will be entered into a restricted partition of the electronic database and remain inaccessible to the follow-up teams.
- **Clinical Staff:** Doctors and nurses at the HNSM who manage pediatric admissions and the BHP team that collects data on admissions and outcomes at the pediatric ward will remain blinded to the study group of any trial participant.
- **Outcome Matching:** The process of matching hospital records to trial participants will be conducted using demographic data by blinded investigators.

6.5.3 Unblinding Procedures and Data Analysis

- **Emergency unblinding:** Individual unblinding will only occur in the event of a medical emergency or a suspected SAE where knowledge of the treatment is critical for clinical management. These instances must be documented and reported to the Lead Investigator and the DSMB.
- **Trial statistician:** The senior trial statistician will remain blinded to the randomization allocation during data cleaning and the final analysis until the DSMB authorizes unblinding.

Justification for Modified Blinding

The implementation of a traditional double-blind protocol - requiring the masking of vials or a "two-person" (preparer and injector) administration chain - is considered operationally unsustainable by the trial team for the following reasons:

1. **Physical environment and crowding:** The delivery and postpartum wards at the study sites primarily consist of high-density, multi-bed ward rooms housing 5 to 10 mothers and their newborns in shared spaces. The cramped bedside areas cannot accommodate the additional research personnel required for a two-person blinding protocol without creating logistical bottlenecks, increasing the risk of needle-stick or administrative errors, and potentially delaying vaccination procedures.
2. **Logistical strain of maternal screening:** The mandatory maternal HBsAg screening introduced in this protocol version adds a significant layer of logistical complexity. Managing rapid diagnostic testing, results communication, and targeted prophylaxis for exposed infants requires dedicated staff time and focus. Combining this intensive screening workload with

the requirements of double-blinding would create an unsustainable burden on the inclusion teams, potentially compromising the integrity of the screening process and delaying the vaccination and discharge of mothers and their newborns, thereby impeding patient flows at the maternity ward.

3. **Clinical necessity of rapid vaccination:** To adhere to WHO recommendations for hepatitis B birth dose administration as soon as possible after birth (ideally <24 hours), the trial prioritizes immediate vaccination over the logistical delays inherent in double-blinding (e.g., off-site pharmacy-based masking). Any procedural delay introduced by masking requirements would withhold the benefit of rapid protection and reduce the real-world clinical relevance of the trial's findings.

By maintaining the blinded status of the parents, follow-up teams, and statisticians, the trial effectively mitigates the major risks of ascertainment and reporting bias while respecting the operational realities of the maternity wards and prioritizing the health of the newborns.

6.6 Breaking of the Study Blinding

The trial statistician will be blinded until the DSMB decides to unblind the trial.

6.7 Subject Withdrawal

Because HBV0 is given in a single dose, the parents will not be able to withdraw their child from treatment except within the short time interval between randomization and vaccination. Consent can be withdrawn for follow-up procedures. Participants whose parents withdraw consent for their child's data to be used will not count towards the total number of participants, and they will not receive further follow-up calls or home visits.

6.8 Protocol Violations

Protocol violations will be recorded throughout the trial for participants in both treatment arms; they will be recorded in a trial log along with all other major events (e.g., health care system strikes, milestones, vaccine campaigns, and any other important observations).

6.9 Trial Closure

In principle, the trial is powered to be completed when 7,250 eligible newborns have been enrolled in each arm of the trial. In practice, enrollment will continue until the Health Ministry of Guinea-Bissau introduces universal HBV0 or when a maximum of 25,000 eligible newborns (12,500 per group) has been enrolled, whichever occurs first.

7 ADVERSE EVENTS

BCG can cause axillary lymphadenitis, which very rarely can ulcerate. Very rarely, in an infant with severe immunodeficiency, BCG can cause disseminated infection (*BCGitis*). OPV0 can cause vaccine-associated paralytic poliomyelitis, diarrhea, fatigue and muscle weakness. For HBV0, the most common reactions are mild soreness, erythema, induration, fatigue, fever, malaise, and influenza-like symptoms. Less common systemic reactions include nausea, vomiting, diarrhea, abdominal pain, abnormal liver function tests, arthralgia, myalgia, rash, pruritus, urticaria.[73] All listed adverse events are uncommon and consistent with the known safety profiles of the vaccines. All three vaccines can cause allergic reactions in very rare circumstances. Each inclusion team will have adrenalin available along with guidelines for medical therapy to handle an acute anaphylactic reaction. All adverse events are captured through the outcome data collection.

8 STATISTICAL METHODS

8.1 Population to be Analyzed

Data analysis will be conducted as intention-to-treat including all participants as randomized, apart from participants whose parents have withdrawn consent for their child's data to be used as part of this study, who will be excluded from analysis.

8.2 Statistical Analysis

The Statistical Analysis Plan (SAP) will be drafted by the trial group and submitted to the DSMB for approval. Upon approval and before initiation of data analysis, the SAP will be uploaded to an online repository. When requested by the DSMB, the trial data can be unblinded, analyzed and reported according to the SAP.

The primary endpoint for the trial will be the composite outcome of all-cause mortality and first non-fatal admission with infection up to 42 days of age with censoring at the time of an event, loss to follow-up, or by 42 days of age, whichever comes first. Cumulative curves for mortality and hospitalization risk will be computed using the mean cumulative function, overall, and by sex. Person-years of risk will be calculated from enrollment (day, hour and minute of randomization). The risk of the single-event composite outcome (all-cause mortality or first non-fatal admission with infection) will be estimated using Cox proportional hazards models with age as the underlying time scale, thus inherently adjusted for age. The Cox models will provide hazard ratios (HRs) from single-event analyses of the composite outcome (all-cause mortality or first non-fatal admission with infection) up to 42 days of age (primary endpoint). Incidence rate ratios for secondary endpoints relating to long-term follow-up for mortality and admissions will be estimated using recurrent-event Andersen-Gill Cox proportional hazards models with robust standard errors. Infants will not be considered at risk of admission while admitted. Randomization will be stratified by sex and place of enrollment in blocks of 24; we will therefore include different underlying baseline hazard functions by sex and place of enrollment in the Cox models. The Cox regression models will have age as the underlying time scale and account for the survival interdependency among twin pairs by using the Stata cluster command. Tests for proportionality of hazard rates will be computed using Schoenfeld residuals, and by visual inspection of cumulative risk curves drawn using the Kaplan-Meier method.

In-hospital case-fatality risk will be estimated using binomial regression providing risk ratios (RRs), also using the Stata cluster command. In case there are fewer than five events in one group, Fisher's exact test will be used to compute a p-value.

Cognitive scores will be standardized and tested using linear regression models.

The cumulative incidence of atopic dermatitis will be separately investigated for self-reported cases and physician-diagnosed cases using binomial regression providing RRs, also using the Stata cluster command.

All analyses will be done overall and by sex using Stata software (StataCorp, College Station, Texas), with all estimates reported with 95% confidence intervals.

8.3 Subgroup Analyses

For mortality and morbidity outcomes, there will be assessment of outcomes by other age groups and of outcomes assessed in more specific ways:

- The composite outcome of all-cause mortality and first non-fatal admission with infection by 42 days of age and by 2 years of age

- All-cause mortality by 42 days of age and 2 years of age.
- In-hospital case-fatality for all-cause and infectious disease admissions by 42 days of age and 2 years of age.
- Morbidity: hospital admissions and in-hospital case-fatality rate for major diseases groups (sepsis, respiratory infections, bronchitis, diarrheal diseases, malaria, malnutrition-related diseases) by 42 days of age and by 2 years of age.

Potential effect modification

Based on literature-identified potential effect modifiers, we will assess whether the effect of HBV0 is modified by the following potential effect modifiers (in addition to sex): vaccination campaigns, the BCG scar status of the mother at enrollment, maternal HIV status, inclusion weight ($\geq 2,500$ g, between $\geq 2,000$ g to $< 2,500$ g, and $< 2,000$ g), season, mode of delivery (vaginal or Caesarean section) and whether the baby was born at a hospital or not. We will conduct a supplementary analysis to assess HBV0's effects by age at enrolment (within 24 hours and ≥ 24 hours after birth), our hypothesis being that HBV0 will have the most marked effects among the youngest neonates. For the composite outcome by 42 days of age and by 2 years of age, we will calculate the female/male ratio.

9 DATA MANAGEMENT

9.1 Data Collection

Data collection will be undertaken by the researchers and entered in trial databases by data entry clerks at the BHP.

9.2 Data Storage

Data will be stored on site-specific departmental password-protected computer files and analyzed in de-identified but re-identifiable form. The de-identified trial database used by the trial statistician to conduct the final data analysis of the trial will be uploaded to a public repository at the time of paper publication.

9.3 Study Record Retention

The Investigator shall maintain all essential trial documents and source data. All records - including signed Informed Consent Forms, Case Report Forms (CRFs), and the Investigator Site File - will be archived in a secure, fire-protected location for a minimum of 20 years following the completion or discontinuation of the study. No records shall be destroyed without prior written authorization from the Sponsor. If the Investigator can no longer maintain the records (e.g., due to retirement or site closure), custody must be transferred to an appropriate person or facility, and the Sponsor must be notified in writing of the new location.

10 ADMINISTRATIVE ASPECTS

10.1 Confidentiality

The Investigator must ensure that the privacy of all study participants is maintained in accordance with applicable data protection laws. All reports and data communication will identify participants only by a unique Subject Identification Code. Particular care will be taken to ensure the confidentiality of sensitive information, including results from maternal HIV testing, with restricted access and secure handling procedures in accordance with applicable data protection standards.

By signing the Informed Consent Form, participants authorize monitors, auditors, and regulatory authorities to access their source medical records for data verification purposes. All study results intended for publication will be strictly anonymized.

10.2 Protocol Amendments

Potential necessary modifications of the trial protocol will be communicated to the DSMB and sent for ethics approval prior to implementation.

10.3 Participant Reimbursement

There will be no reimbursement for the participants.

10.4 Financial Disclosure and Conflicts of Interest

None of the investigators have a financial disclosure or conflict of interest to disclose.

11 POST-TRIAL ACCESS AND KNOWLEDGE TRANSLATION

The HBV-NSE trial is conducted within a defined "window of opportunity" preceding the planned expected 2028 national introduction of HBV0 in Guinea-Bissau. The investigators are committed to ensuring that the knowledge generated by this study translates into immediate public health benefits and informs the national immunization strategy.

Rapid Data Dissemination and Transparency

Because trial data - including mortality and morbidity outcomes - are collected and digitized in real-time via the BHP's established surveillance infrastructure, the trial is uniquely positioned for rapid analysis and dissemination. The investigators are committed to make the primary trial results available to the Guinea-Bissau Ministry of Health (MoH) and the National Immunization Technical Advisory Group (NITAG) within three months of the cessation of enrollment, provided that the DSMB has unblinded the trial data.

Global Data Sharing: The trial team will upload summary results and trial data to ClinicalTrials.gov and other appropriate open-access repositories. This ensures that the findings are shared quickly with the global scientific community and the WHO SAGE.

Local Stakeholder Engagement

Upon the conclusion of the trial and submission of the primary trial results to the MoH and NITAG, the study team will organize a dissemination seminar in Bissau for representatives from the MoH, the Expanded Programme on Immunization (EPI), the NITAG, and HNSM leadership. This forum will ensure that local decision-makers are the first to be informed of the trial's implications for neonatal health and can use the findings to optimize any planned rollout of HBV0 in Guinea-Bissau.

Implementation Support via Urban and Rural Teams

The BHP's extensive network of urban and rural surveillance and vaccination teams represents a unique resource for the Guinea-Bissau health system. When or if the decision for universal HBV0 implementation is made, these teams can assist the Ministry of Health in a nationwide rollout.

12 USE OF DATA AND PUBLICATIONS POLICY

The trial results will be published in peer-reviewed journals. A summary in Portuguese and a copy of the trial report will be sent to the library of the National Institute of Health in Guinea-Bissau (INASA), and the results will be communicated to the National Committee of Ethics in Guinea-Bissau.

13 ETHICAL CONSIDERATIONS

With ~18 out of 47 WHO African region countries having implemented HBV0[61] and vaccination coverage being low[74], the present trial addresses a major public health question in Guinea-Bissau and the region, where hepatitis B is highly prevalent[34,35] and national policies regarding HBV0 are likely to change in the coming years. The overall health effects associated with the addition of HBV0 to the vaccination schedule, including when co-administered with other neonatal vaccines, have not previously been evaluated in randomized studies and remain an area where further randomized evidence has been called for.[75]

There is no uncertainty as to whether HBV0 prevents vertical transmission in the neonatal period. For newborns born to HBsAg-negative mothers, however, the direct specific benefit of HBV0 is expected to be absent or minimal. The overall health effects of providing HBV0 with BCG and OPV0 remain unknown. Particularly in newborns born to HBsAg-negative mothers, there is genuine uncertainty as to whether universal HBV0 administration confers net benefit, has no overall effect, or even negative effects. Immunological studies have shown that HBV0 administration at birth can alter neonatal immune responses in ways that remain insufficiently characterized in terms of clinical outcomes.[47] In high-income countries with a low prevalence of hepatitis B and with no logistical or financial barriers impeding implementation of newborn vaccines, many countries have chosen not to implement universal HBV0. In The Gambia, where HBV0 is universally implemented, ethics committees permitted delay in children of HBsAg-negative children in association with a clinical trial.

These considerations support the ethical and scientific rationale for evaluating the broader health effects of HBV0 prior to universal policy implementation in Guinea-Bissau, by comparing the current policy of BCG + OPV vs the coming policy of BCG + OPV + HBV0 in children of HBsAg-negative mothers.

Noteworthy, not conducting such a trial may carry important public health and scientific implications, including continued uncertainty regarding the overall health effects of HBV0 and the optimal strategy for its implementation. The results of the trial are therefore expected to help inform both national and regional vaccination policies. See also Supplementary tables 1 and 2.

Maternal screening for HBsAg and HIV-1/HIV-2 will be performed prior to enrolment, with separate informed consent for each test. Newborns born to mothers known or found to be HBsAg-positive are excluded from randomization and are offered HBV0 immediately, reducing the risk of vertical transmission.[76,77] In addition, maternal HIV testing with counselling and referral is incorporated to facilitate early identification and linkage to care for mothers and newborns, improving access to preventive and treatment services. The offer of HBV0 vaccination to infants not enrolled in the trial is an expansion of access to preventive services enabled by the trial platform, rather than a reflection of the routine standard of care, which is BCG + OPV0 vaccination at birth. The randomized comparison therefore remains between the current national vaccination schedule (BCG + OPV0) and the addition of HBV0 under controlled conditions, while the trial platform at the same time minimizes the risk of vertical transmission of hepatitis B virus.

The trial is thus designed to ensure direct benefits and minimize risks to participants. All participants receive BCG and OPV0 in accordance with national guidelines, and the trial strengthens access to timely vaccination, structured follow-up, and clinical care. Free consultations at three health centers and essential medications are made available to all newborns approached for enrolment, regardless of participation. Together, these measures provide direct benefits to both participants and non-participants, improve access to care, while incorporating additional safeguards for both participating newborns and those who decline participation or are ineligible.

From a scientific and ethical perspective, randomized allocation is necessary because the trial addresses a question for which no reliable answer currently exists; whether adding universal HBV0 to BCG + OPV0 among newborns born to HBsAg-negative mothers improves or worsens overall child health outcomes. While HBV0's benefit in reducing perinatal hepatitis B infection is established, the absolute risk at the population level is relatively low, with the benefit primarily concerning a relatively small subgroup of infants to whom hepatitis B virus may be transferred vertically.

A randomized design is required to generate robust evidence to inform policy decisions and reduce skepticism. Notably, the need for randomized trials to evaluate the potential non-specific effects of vaccines has been recognized in international policy discussions, including deliberations within WHO's SAGE, who highlighted the importance of rigorous experimental designs to assess broader health outcomes beyond disease-specific protection.[75,78]

The choice of an individually randomized design with allocation at birth reflects both scientific and operational considerations. The study will be conducted in a high-volume maternity setting with many daily deliveries, where swift enrolment and vaccination are required. A double-blind design would not be operationally feasible under these conditions without compromising enrolment, increasing complexity, or potentially delaying vaccination. The use of a more simple and pragmatic design allows efficient implementation while maintaining the necessary scientific rigor.

The trial team will implement measures to minimize bias: randomization is performed using pre-specified allocation procedures, and outcome assessment and data analysis are conducted blinded to group allocation. The use of objective outcomes, including mortality and hospital admissions caused by infection, further reduces the risk of bias.

The trial will be conducted in adherence to the ethical standards outlined in the Declaration of Helsinki. The revised trial protocol was submitted to the ethical committee of Guinea-Bissau, and approved (approval number 06-CNEPS-INASA-2026). It was not possible to submit the protocol to the National Ethics Committee in the Sponsor country (Denmark) due to the current Danish law governing research ethics committees. For this reason, the protocol was submitted to the Research Ethics Committee of the University of Southern Denmark (SDU REC). On 30 September 2026, SDU REC issued a positive advisory assessment (Case no. 26/55358 / REC762). Pursuant to the decision of SDU's Executive Board of 16 April 2026, SDU REC assessments are advisory. The formal approval to proceed with the revised hepatitis B study was taken by the Dean of the Faculty of Health Sciences on October 7, 2026.

Information about the trial will be provided in clear, understandable language considering the cultural and educational background of the mother/guardian, and the difficulties surrounding childbirth in Guinea-Bissau. It will be ascertained that parents or guardians fully understand the implications of trial participation.

It will be explained that all invited neonates (regardless of consent to participate) are entitled to free consultations during infancy at the three health centers located within the HDSS of the Bandim Health Project, in Bandim, Belém and Cuntum. Children presenting for consultation will be given

access to essential medications free of charge. Participants in the trial will be covered via the collateral insurance agreement of the University of Southern Denmark.

For the subgroup of children participating in the neurodevelopmental assessment, it will be emphasized that the results can help identify a child's strengths and areas where they might need additional support. Testing can help identify potential learning difficulties early on, allowing for timely interventions and support. It will be explained that cognitive tests are not the sole measure of a child's intelligence or potential. They provide a snapshot of specific cognitive skills at a particular time. Parents will be reassured that there is no need for children to prepare for the tests, and that the aim is to understand their current abilities, not to catch them out. It will be made clear that results are kept strictly confidential.

For the subgroup of children participating in the assessment of AD (due to an itchy rash), they will have access to a study MD with specialized training in diagnosing and treating atopic AD.

We will use a HBV0 vaccine that has been evaluated and approved by WHO (pre-qualification) to meet international standards of quality, safety and efficacy.

HNSM is currently the only Maternity Center in urban Bissau where BCG and OPV0 are routinely administered daily, although not on weekends and public holidays. As a consequence of the trial, more children will have timely vaccination opportunities, as BCG and OPV0 vaccines will be provided at all recruitment centers on each weekday. Vaccination of these newborns would otherwise often be delayed and procuring vaccination in Guinea-Bissau can be associated with a financial burden for the household.[79,80] Given that RCTs indicate beneficial non-specific effects of both BCG[1] and OPV0[16], all participants are thus expected to benefit from participating in the trial. Whether the trial identifies that HBV0 is associated with positive, negative or no non-specific effects, it will be of high importance for global vaccine policy, providing crucial input on how to best reduce child mortality and morbidity. The trial will provide local and global health authorities and immunization stakeholders updated data of the burden of hepatitis B disease in Guinea-Bissau and the overall health effects associated with HBV0. Such data will be the best way to maintain vaccine confidence in the long run.

14 APPENDIX

14.1 Trial Inclusion Form.

Regno (birth number, HNSM): ____ Adhesive study number: ____ Written study number: ____
Date ____/____/20__ Inclusion assistant: _____ Place of enrollment: _____
The child already received a vaccine: Yes__ No__ Is sick: Yes__ No__ Age ≤ 1 week: Yes__ No__
Congenital malformation: Yes__ No__ Informed consent to participate in the trial: Yes__ No__
Informed consent to maternal HBsAg test: Yes__ No__ HBsAg test result: Positive__ Negative__
Confidential counselling provided for HBsAg-positive result: Yes__ No__
Can be included: Yes__ No__
Informed consent to maternal HIV-1/HIV-2 test: Yes__ No__
Consent for follow-up contact regarding HIV test results and related care: Yes__ No__
HIV test result (if performed): Positive (HIV-1)__ Positive (HIV-2) Negative__ Indeterminate__
Confidential counselling provided for HIV-positive result: Yes__ No__
Referred for confirmatory testing & linkage to HIV care services (if HIV test positive): Yes__ No__

Information about the parents

Relation to the child ____ (1: Mother, 2: Father, 3: Uncle/aunt, 4: grandparent, 5: Other)
The mother is alive: Yes__ No__ If no, date of death: ____/____/202__
Mother's name _____
Telephone number, Orange: _____ Telephone number, Airtel: _____
Does not have a telephone__ Does not remember telephone number__ House name _____
Age____ Ethnicity____ Schooling____ From BHP area Yes__ No__ BHP address
information: Neighborhood and zone (TaBz)____ House number (CaMo)____ Child ID
(CNO)____ Neighborhood (if not BHP area) _____
Mother's place of birth _____
Father's name _____
Telephone number, Orange: _____ Telephone number, Airtel: _____
Does not have a telephone__ Age____ Ethnicity _____ Went to school
Yes__ No__ Unknown__ Father lives with mother Y__ N__
Other contacts and relation to the child
Name contact 1 _____ Relation _____ N° _____
Name contact 2 _____ Relation _____ N° _____

Name contact 3 _____ Relation _____ N° _____

Measurements

Mother: Arm circumference _____ mm Has BCG scar Yes ___ No ___ Width _____ Height _____ Assistant _____

Father: Arm circumference _____ mm Has BCG scar Yes ___ No ___ Width _____ Height _____ Assistant _____

Information about the child

Name (if named) _____ Birth date _____ / _____ /202 _____

Time of birth ____:____ birthplace _____ C-section Yes ___ No ___

Sex Male ___ Female ___ Twin Yes ___ No ___ Twin number _____ Twin study number _____

Height _____ Head circumference _____ Admitted before enrolment Yes ___ No ___ Where _____

If admitted, date of hospital admission ____/____/20__ Date of discharge ____/____/20__

Birth weight _____ Weight on day of inclusion _____

Confirmed the child's sex (assistant name): _____

The mother or guardian of the child provided consent for participation in the trial: Yes ___ No ___

Observations _____

Randomization

Date of randomization ____/____/202__ Time of randomization ____:____

Randomization lot number _____

Randomization result: HBV0 _____

Randomization result: Placebo _____

Date of **HBV0/placebo** vaccination: ____/____/202__ Time ____:____ Batch **HBV0**: _____ Expiry ____/202__

Date of **BCG** vaccination: ____/____/202__ Time ____:____ Batch **BCG**: _____ Expiry ____/202__

Date of **OPV0** vaccination: ____/____/202__ Time ____:____ Batch **OPV0**: _____ Expiry ____/202__

BCG vaccination papule: Height ____ mm Width ____ mm Vaccinator: _____

BCG vaccine strain administered: ____ (Denmark: 1, Japan: 3, Bulgaria: 5, Russia: 8)

Confirmed by: _____

14.2 Definition of hospital admission with infection (fatal and non-fatal)

The definitions below specify how components of the primary outcome are identified in the BHP HNSM Pediatric Ward database.

The following discharge codes for the variable **smxcau** define the vital status of the child:

- 51 Discharged alive
- 53 Died during admission
- 54 Left (fuga) – documented carefully and rarely used
- 55 Admitted, section and bed known
- 56 Admitted, location unknown
- 57 Transferred to other hospital
- 59 Missing information

The variable **regdate** defines the day of admission, and the variable **saida** defines the day of discharge.

Outcome definition

In-hospital death: If a child has an **smxcau** variable code of 53, the child died during admission, with the date of death being the date defined in **saida**. All admissions with an **smxcau** code of 53 where the death occurred before 42 days of age (**saida** date lies within 42 days after birth) will count towards the primary outcome (composite outcome of death or non-fatal admission with infection before 42 days of age). Infants with all other codes than 53 will be considered as having survived the admission.

Non-fatal admission with infection: Infants that were admitted before 42 days of age that survived the admission (**smxcau** code 51) and infants admitted before 42 days of age that died after 42 days of age will count towards the primary outcome, provided that the hospital admission was caused by an infection. Infection will be defined using the definitive diagnosis code defined in the variable **cddefdiag1** attributed by the hospital's treating physicians (if attributed), and if no definitive diagnosis code was attributed, then the admission diagnosis code **cdprodiag1** will be used.

The following diagnosis codes attributed in the HNSM pediatric ward database of hospital admissions will be counted as hospital admissions caused by infection:

Pneumonia, diagnosis code = 2

Convulsions, diagnosis code = 3, is included as an infectious admissions due to their frequent association with underlying infections in this setting.

Gastroenteritis, diagnosis code = 5

Malaria, diagnosis code = 6

Measles, diagnosis code = 7

Fever, diagnosis code = 10, is included as an infectious admission due to their frequent association with underlying infections in this setting.

Meningitis, diagnosis code = 12

Tetanus, diagnosis code = 13

Bronchitis, diagnosis code = 14

Sepsis, diagnosis code = 15

Chickenpox, diagnosis code = 23

Intestinal parasitosis, diagnosis code = 24

Urinary infection, diagnosis code = 26

Syphilis, diagnosis code = 27

Polio, diagnosis code = 31

Neonatal tetanus, diagnosis code = 32

Pertussis, diagnosis code = 34

TB (ex-pulmonary), diagnosis code = 35

Lepra, diagnosis code = 36

Dysentery, diagnosis code = 37

Cholera, diagnosis code = 38

Rabies, diagnosis code = 39

Other respiratory disease including pulmonary TB, diagnosis code = 48

HIV/AIDS, diagnosis code = 52

For hospital admissions with the diagnosis code 19 (Other) or code 20 (No pathology defined), categories given to rarer causes of admission not defined in the above coding, a string variable (defdiag1, if not available, then prodiag1) may define the cause of admission. In such cases, the admission will be defined as caused by an infection if the string variable contains one of the following diagnoses (text strings): Abscess, bronchitis, candidiasis, cellulitis, conjunctivitis, whooping cough (convulsa, pertussis), impetigo, stomatitis, furunculitis, O.M.A (acute otitis media), mastitis, omphalitis, "catarro comun" (upper airway infection), febrile syndrome, meningoencephalitis, shock or "shock misto" (septic shock). For diagnosis codes 19 and 20, classification is based on the string diagnosis variable; if no relevant infectious term is present or the string variable is missing or does not contain interpretable information, the admission will be classified as non-infectious. Skin disorders, diagnosis code = 21 will be classified as non-infectious unless a text string diagnosis indicates an infectious condition (e.g., impetigo, cellulitis, abscess).

Non-fatal admission due to non-infectious causes

The below hospital diagnosis codes will be defined as not being an infection:

Anemia, diagnosis code = 1

Dehydration, diagnosis code = 4

Vomiting, diagnosis code = 8

Malnutrition, diagnosis code = 9

Cardiac insufficiency, diagnosis code = 11

Prematurity, diagnosis code = 16

Nephrotic syndrome, diagnosis code = 17

Distressed newborn, diagnosis code = 18

Other, diagnosis code = 19 (see exemption above)

No pathology defined, diagnosis code = 20 (see exemption above)

Skin disorders, diagnosis code = 21 are classified as non-infectious unless the string diagnosis indicates an infectious condition (e.g., impetigo, cellulitis, abscess).

Trauma, diagnosis code = 22

Intoxication, diagnosis code = 25

Hypoglycemia, diagnosis code = 28

Icteric syndrome, diagnosis code = 33

Hypothermia, diagnosis code = 40

Congenital cardiac condition, diagnosis code = 41

Congenital malformation, diagnosis code = 42

Acquired cardiac disease, diagnosis code = 43

Epilepsy or cerebral palsy, diagnosis code = 44

Sequelae from spino-cerebral infections, diagnosis code = 45

Other neurologic/psychiatric disorder, diagnosis code = 46

Asthma, diagnosis code = 47

Malabsorption, diagnosis code = 49

Rheumatic disease, diagnosis code = 50

Other bodily disease, diagnosis code = 51

Other chronic disease, diagnosis code = 53

Leukemia, diagnosis code = 54

Other malignant disease, diagnosis code = 55

Sequelae from trauma, diagnosis code = 56

No information, diagnosis code = 99

14.3 Response to external ethical and scientific feedback

1. Concern raised: Withholding HBV0 is unethical because HBV0 has proven benefit and foreseeable harm if not given

From an ethical perspective, the first version of the protocol did not remove or withhold any vaccines included in the national immunization schedule; all newborns would receive BCG and OPV0 in accordance with the current national schedule in Guinea-Bissau. In the revised protocol, we have added maternal HBsAg screening prior to enrolment. Neonates born to mothers known or found to be HBsAg-positive will be excluded from randomization and offered HBV0. This amendment was introduced to ensure that newborns at risk of vertical hepatitis B transmission are identified and offered birth dose vaccination, while allowing the trial to evaluate the overall health effects of universal HBV birth dose implementation, which is essential because for newborns born to HBsAg-negative mothers, HBV0 does not confer direct benefit in preventing vertical transmission.

2. Concern raised: there is no scientific necessity for a no-treatment / no-vaccine control arm

From a scientific perspective, the rationale for randomized evaluation of NSEs has previously been acknowledged in WHO's own review of NSEs, which concluded that conclusive evidence regarding effects of vaccines on mortality is unlikely to be obtained from observational studies alone.[45,78] The need for more high-quality randomized controlled trials where feasible was thus highlighted. WHO's SAGE subsequently worked over several years on the development of protocols for trials to test NSEs.[75]

The present investigator-initiated trial addresses this unresolved question regarding potential NSEs associated with universal hepatitis B birth dose vaccination in Guinea-Bissau. As documented by WHO's SAGE resolving this question methodologically requires a no-vaccine control arm while protecting infants at highest risk of vertical transmission through maternal HBsAg screening and targeted neonatal vaccination.

3. Concern raised: insufficient scientific justification; no credible evidence of a safety signal

There is both observational data from the region (Guinea-Bissau[48] and The Gambia[49]) suggesting that hepatitis B vaccination in infancy may be associated with sex-differential NSEs, and recent randomized trial evidence from The Gambia indicates differential immune responses associated with different newborn vaccination schedules.[47]

However, the primary scientific rationale for the present trial is not the presence of a confirmed safety signal, but the absence of randomized evidence on the broader health effects of HBV0 when administered at birth, either alone or in combination with BCG and OPV0. Given the widespread use of HBV0 and its potential to influence early-life immune responses, this represents a critical evidence gap. As highlighted by WHO's SAGE, randomized trials are necessary to provide conclusive evidence on the overall health effects of vaccines beyond pathogen-specific protection to guide policy changes.[75]

4. Concern raised: biased / low-utility design

A placebo was not included in the prior version of the protocol because the ethical committee in Guinea-Bissau had advised against unnecessary injections; the revised trial protocol will be submitted to the ethical committee for renewed assessment. The revised trial protocol will be implemented as a placebo-controlled, participant-blinded trial with blinded outcome assessment and

blinded statistical analysis. This addition of a saline placebo was introduced specifically to reduce the risk of ascertainment and reporting bias raised in the WHO statement. In our setting, it is not operationally feasible to conduct a fully double-blind randomized controlled trial. Enrolment occurs in a busy maternity ward where a high number of newborns must be enrolled each day shortly after delivery, often within a narrow time window when both mother and infant are still present at the facility. Implementing a double-blind design would require pharmacy preparation, labeling, and controlled allocation of masked vaccine and placebo vials, as well as additional personnel and procedures that would substantially slow enrolment and risk delaying administration of routine neonatal interventions. The trial will therefore instead employ a participant-blinded design with blinded outcome assessment and blinded statistical analysis, which preserves protection against major sources of bias while remaining feasible within the clinical workflow of high-volume maternity settings.

5. Concern raised: “exploiting scarcity is not ethical”

Neither the first proposed trial or the revised trial protocol merely replicates routine care. On the contrary, it expands access to preventive and clinical services that are not always available to newborns in Guinea-Bissau. The trial will support timely neonatal BCG and OPV0 vaccination, includes structured follow-up, and independent DSMB oversight. In addition, all newborns approached for enrolment in the study, regardless of whether they participate, will be offered access to free consultations during infancy at participating health centers and essential medications free of charge. These measures are intended to minimize risk, reduce financial barriers to essential care, and increase direct participant benefit.

In addition, the revised protocol adds maternal HBsAg screening measures with targeted, immediate prophylactic action for exposed newborns, which is not currently part of newborn care in Guinea-Bissau. HBV0 vaccination will be made available at the participating facilities to all newborns born to HBsAg-positive mothers and all that are ineligible to participate for other reasons, including families who choose not to participate in the trial. In this way, the study establishes access to preventive services that are not currently available as part of routine newborn care.

By introducing maternal hepatitis B screening, expanding access to HBV prevention, strengthening routine neonatal vaccination, and providing free consultations and essential medicines, the study contributes to improving local standards of care and safeguards both participants and non-participants. Rather than exploiting scarcity, the trial seeks to reduce gaps in access to prevention and clinical care while generating evidence needed to inform national and global vaccination policy, without disadvantaging participants or non-participants.

6. Concern raised: the protocol does not ensure a minimum level of harm reduction, such as screening pregnant women and vaccinating exposed newborns

In the first version of the protocol, maternal HBsAg screening was not included because the trial was designed as a pragmatic evaluation of introducing the HBV0 under real-world conditions in Guinea-Bissau, where neither HBV0 nor universal maternal hepatitis B screening is part of routine care. Adding universal maternal screening constitutes a substantial additional intervention that alters the research question from *evaluating population-level introduction of HBV0* to *evaluating vaccination conditional on prior screening*. It also entails requirements for counselling, disclosure, referral, and follow-up that are not routinely available in maternity settings and may affect feasibility, timeliness of enrolment, and representativeness of the study population. The initial design therefore aimed to assess a realistic and scalable implementation strategy aligned with current health system conditions.

We nevertheless accept this criticism, also in light of the latest revisions included in the Helsinki Declaration, and have revised the protocol accordingly.

Maternal HBsAg screening will be implemented prior to enrolment, with separate informed consent for testing. Maternal HIV-1/HIV-2 testing will also be offered with separate informed consent, with provision of confidential counselling and referral for confirmatory testing and linkage to HIV care services for mothers with a positive screening test. Infants of mothers known or found to be HBsAg-positive will be excluded from randomization and offered HBV0 promptly, with counselling provided to the mother by a medical doctor or trained counsellor. This amendment directly addresses the concern that the prior protocol did not sufficiently protect newborns at risk of vertical transmission.

14.4 Supplementary Tables

Supplementary Table 1. Public health and scientific consequences of not conducting the HBV-NSE trial

Domain	Consequence if the trial is not conducted	Implications
Evidence for HBV0 policy	Uncertainty regarding the overall health effects (beneficial, neutral, or adverse) of universal HBV0 administration	Policy decisions will continue to rely on incomplete evidence, potentially leading to suboptimal implementation strategies
Vaccine policy in SSA	Lack of context-specific evidence on whether universal or targeted HBV0 strategies are most appropriate	Health authorities may adopt strategies not optimized for local epidemiology and health system context
Understanding of vaccine interactions	Limited knowledge on how HBV0 interacts with BCG and OPV0 when co-administered at birth	Missed opportunity to optimize neonatal vaccine schedules and combinations
Health system practice at birth	Continued variability in timing and delivery of birth vaccines without evidence on optimal strategies	Ongoing heterogeneity in care without evidence-based guidance
Access to timely birth vaccination	Missed opportunity to ensure timely administration of BCG and OPV at birth within the study setting (e.g., in weekends and holidays)	Continued gaps in timely neonatal vaccination delivery at HNSM's maternity ward
Maternal screening and targeted prevention	Lack of systematic identification of HBsAg-positive mothers and targeted HBV0 vaccination of high-risk newborns in the study cohort	Missed opportunity to strengthen prevention of vertical transmission in the study setting
Capacity building and local research development	Lost opportunity for training and capacity strengthening of local researchers, including PhD-level training of two Bissau-Guineans	Slower development of sustainable local research capacity and leadership

Supplementary Table 2. Interpretation of possible trial outcomes and potential policy implications

Trial result	Interpretation	Policy implications
HBV0 has beneficial non-specific effects (reduces morbidity/mortality)	Suggests HBV0 confers broader immunological benefits in early life, potentially enhancing resistance to unrelated infections when co-administered with BCG and OPV0, consistent with emerging concepts such as trained immunity	Supports universal HBV0, also in low-prevalence settings; strengthens rationale for co-administration strategies; encourages further research into mechanisms of beneficial NSEs
HBV0 has no measurable non-specific effects	Indicates that HBV0 primarily provides pathogen-specific protection without broader effects on overall health outcomes	Supports current policy decisions based on hepatitis B epidemiology alone and that co-administration with BCG and OPV0 is neutral with respect to overall health
HBV0 has adverse non-specific effects (increases morbidity/mortality)	Suggests that HBV0 may adversely modulate early-life immune responses in certain contexts or when co-administered with BCG + OPV	Reassessment of HBV0 policies, particularly in low-risk populations. Consider targeted HBV0 strategies to high-risk newborns.

Supplementary Table 3. Overview of global neonatal trials with the level of background care including neonatal vaccines provided

Trial program	Years conducted	Age at inclusion	WHO involvement	Background care provided to participants	Was HBV birth dose provided to participants?
NeoVita trials (Ghana, Tanzania, India) [81–83]	2010–2014	≤72 hours after birth	WHO coordinated the multi-country research program following a technical consultation and call for trial sites.	Routine newborn care and immunizations were provided through national health services according to local policy. The trial intervention consisted only of vitamin A or placebo.	Not provided. The HBV birth dose was not provided through the trial. In India it was national policy and could be received through routine services; in Ghana and Tanzania the HBV birth dose was not part of the national immunization schedule at the time.
AFRINEST trials (DR Congo, Kenya, Nigeria) [84]	2011–2014	0–59 days of age	WHO coordinated the multicountry research program evaluating simplified antibiotic regimens for neonatal infection.	Infants received clinical assessment and antibiotic treatment according to the study protocol when referral was not feasible, in addition to routine health services.	Not provided. National immunization schedules in the participating countries generally did not include universal HBV birth dose vaccination during the trial period.
SAR trials (Africa and South Asia) [85]	2011–2014	0–59 days of age	Conducted as part of the WHO-supported research program evaluating simplified antibiotic regimens for possible serious bacterial infection.	Infants received outpatient antibiotic treatment and routine clinical care according to study protocols and local health services.	Not provided. In many participating countries hepatitis B vaccination was provided through pentavalent vaccine starting at about 6 weeks of age.
Chlorhexidine cord care trials (Nepal, Bangladesh, Pakistan) [86–88]	2002–2010	Immediately after birth (less than 24 hours old)	Trials conducted by academic and public health institutions; WHO later reviewed the evidence and issued global recommendations.	Community-based newborn care including home visits by trained study workers for enrolment, cord assessment, and monitoring of neonatal health, in addition to routine local newborn care services.	Not provided in Nepal and Bangladesh; limited or inconsistent use in Pakistan; most participants did not receive HBV birth dose.

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