RANDOMIZED, CONTROLLED, PHASE 1 STUDY TO ASSESS SAFETY AND IMMUNOGENICITY OF CO-ADMINISTERED *NA*-APR-1 (M74)/ALHYDROGEL® AND *NA*-GST-1/ALHYDROGEL® HOOKWORM VACCINES IN GABONESE CHILDREN

Protocol Number: HV-002

IND Sponsor: Baylor College of Medicine

Principal Investigator: Ayola Akim Adegnika, MD, PhD, MSc

Final Version Number: 3.0

Including Amendment #2

08 August 2018

STATEMENT OF COMPLIANCE

The study will be carried out in accordance with Good Clinical Practice (GCP) as required by the International Conference on Harmonisation efficacy guideline E6.

All key personnel (all individuals responsible for the design and conduct of this study) have completed ethical training in the protection of the participants of clinical trials.

SIGNATURE PAGE

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable ICH guidelines.

Site Investigator:

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Title: Principal Investigator

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AE	Adverse Event
AESI	Adverse Event of Special Interest
AIGHD	Amsterdam Institute for Global Health and Development
APR-1	Aspartic Protease-1
CBC	Complete Blood Count
CERMEL	Centre de Recherches Médicales de Lambaréné
cGMP	Current Good Manufacturing Practices
CIOMS	Council for International Organizations of Medical Sciences
CRF	Case Report Form
ELISA	Enzyme linked immunosorbent assay
FDA	Food and Drug Administration
GCP	Good Clinical Practice
GLA-AF	Gluco-Pyranosylphospho-Lipid A Aqueous Formulation
GMP	Good Manufacturing Practice
GST-1	Glutathione S-Transferase
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B virus
hCG	Human choriogonadotropin
HCV	Hepatitis C virus
HHVI	Human Hookworm Vaccine Initiative
HIV	Human Immunodeficiency Virus
IB	Investigator's Brochure
ICF	Informed Consent Form
ICH	International Conference on Harmonisation
IEC	Independent or Institutional Ethics Committee
IM	Intramuscular
IND	Investigational New Drug Application
IP	Investigational Product
IRB	Institutional Review Board
ISM	Independent Safety Monitor
MAAE	Medically-Attended Adverse Event
MedDRA®	Medical Dictionary for Regulatory Activities
MBC	Memory B Cell
MOP	Manual of Procedures
N	Number (typically refers to subjects)
Na	*Necator americanus*
PBMC	Peripheral Blood Mononuclear Cell
PI	Principal Investigator
QA	Quality Assurance
QC	Quality Control
SAE	Serious Adverse Event
SMC	Safety Monitoring Committee
SOP	Standard Operating Procedure
SVI	Sabin Vaccine Institute (Albert B Sabin Vaccine Institute; Sabin)
BCM	Baylor college of medicine
US	United States

WBC	White Blood Cell
WHO	World Health Organization

KEY ROLES

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PROTOCOL SUMMARY

Title:	Randomized, controlled, Phase 1 Study to assess safety and immunogenicity of co-administered Hookworm vaccine candidates *Na*-APR-1 (M74)/Alhydrogel® and *Na*-GST-1/ Alhydrogel® in Gabonese Children
Phase:	1
Population:	Healthy male and female children aged 6-10 years, inclusive.
Number of Sites:	1 (Centre de Recherches Médicales de Lambaréné, Gabon)
Study Duration:	20 months
Subject Participation Duration:	15 months
Description of Interventions:	a) The ***Na*-APR-1 (M74)** candidate vaccine contains recombinant *Na*-APR-1 (M74) adsorbed onto aluminum hydroxide gel (Alhydrogel®) and suspended in a solution containing 10mM imidazole, 150mM sodium chloride and 0.3% Empigen BB, with pH 7.4 ± 0.1. The final concentrations of *Na*-APR-1 (M74) and Alhydrogel® in the drug product are 0.1mg/ml and 0.8mg/ml, respectively. Doses of 10, 30 and 100 µg *Na*-APR-1 (M74) will be delivered IM to the deltoid by injecting different volumes of the 0.1mg/ml preparation. b) The ***Na*-GST-1** candidate vaccine contains the recombinant *Na*-GST-1 adsorbed onto Alhydrogel® and suspended in a solution containing 10mM imidazole and 10% glucose. The final concentrations of *Na*-GST-1 and Alhydrogel® in the drug product are 0.1mg/ml and 0.8mg/ml, respectively. Only one dose of *Na*-GST-1 will be tested (100µg), at a volume of 1.0ml delivered IM to the deltoid. c) Gluco-Pyranosylphospho-Lipid A Aqueous Formulation (**GLA-AF**) is a Toll-like Receptor-4 agonist. Point-of-injection formulations with this immunostimulant will be prepared immediately prior to vaccination by adding an appropriate volume of GLA-AF to *Na*-APR-1 (M74) and withdrawing an appropriate volume to administer the desired amount of *Na*-APR-1 (M74) plus 5µg GLA-AF IM to the deltoid. d) The ENGERIX-B hepatitis B vaccine will serve as the comparator vaccine. Each 0.5 ml dose contains 20 µg recombinant hepatitis B surface protein expressed by

Saccharomyces cerevisiae and adsorbed to aluminum hydroxide will be injected IM to the deltoid.

e) To maintain the study blind by ensuring that all subjects receive two injections at each vaccination point, sterile normal **saline** (0.9%) for injection will be co-administered to those subjects randomized to receive Hepatitis B vaccine.

Objectives:

Primary:

Safety

1. To evaluate the safety and reactogenicity of three different dose concentrations of *Na*-APR-1 (M74)/Alhydrogel® co-administered with *Na*-GST-1/Alhydrogel® in healthy Gabonese children.

Secondary:

Immunogenicity

1. To determine the doses of co-administered *Na*-APR-1 (M74) and *Na*-GST-1 that result in the highest levels of IgG antibody approximately 14 days after the third vaccination.

Tertiary:

Immunogenicity

1. To assess and compare the duration of the antibody responses of *Na*-GST-1 and *Na*-APR-1 (M74).
2. To assess the distribution of IgG subclass responses to *Na*-GST-1 and *Na*-APR-1 (M74).

Exploratory:

1. To assess the cellular immune responses to the *Na*-GST-1 and *Na*-APR-1 (M74) antigens following immunization.
2. To assess the impact of co-administered *Na*-GST-1 and *Na*-APR-1 (M74) on the production of memory B cells specific for each antigen.
3. To assess the production of memory B cells specific for each vaccine antigen compared to metabolomic changes before and after vaccination
4. To characterize the metabolite composition in urine and serum during the course of immunization and assess the changes on metabolite profiles related to the co-administration of *Na*-GST-1 and *Na*-APR-1 (M74).

Description of Study Design:

Double blind, randomized, controlled, dose-escalation Phase 1 clinical trial in hookworm-exposed children aged 6 to 10 years living in the area of Lambaréné, Gabon. Children will receive three doses of the assigned vaccine(s) delivered intramuscularly (deltoid) on approximately Days 0, 56, and 112 or 180.

Safety will be measured from the time of each study vaccination (Day 0) through 14 days after each study vaccination by the occurrence of solicited injection site and systemic reactogenicity events.

Unsolicited non-serious adverse events (AEs) will be collected from the time of the first study vaccination through approximately 1 month after each study vaccination. New-onset chronic medical conditions and Serious Adverse Events (SAEs) will be collected from the time of the first study vaccination through approximately 9 months after the third study vaccination (final visit). Clinical laboratory evaluations for safety will be performed on venous blood collected approximately 14 days after each vaccination.

Immunogenicity testing will include IgG antibody responses to each vaccine antigen, by a qualified indirect enzyme-linked immunosorbent assay (ELISA), on serum or plasma obtained prior to each study vaccination and at time points after each vaccination (see Appendix A); the functional activity of vaccine-induced antibodies will be assessed by *in vitro* enzyme neutralization assays; the induction of B cell memory will be measured by antigen-specific memory B cell responses.

Recruitment and enrollment into the study will occur on an ongoing basis, with each group being recruited and vaccinated in sequence.

60 subjects will be enrolled into 3 groups of 20:

The first 20 subjects will be assembled and enrolled into Group 1:

- Group 1 double-blind IP allocation (n=20):
    - 16 subjects will receive 10 µg *Na*-APR-1 (M74) plus 5 µg GLA-AF delivered by IM injection in the deltoid muscle, with 10 µg *Na*-GST-1 administered IM in the alternate arm.
        - 8 will be vaccinated according to a 0,2,**4**-month schedule
        - 8 will be vaccinated according to a 0,2,**6**-month schedule

- 4 subjects will receive hepatitis B vaccine comparator:
  - 2 will be vaccinated according to a 0,2,**4**-month schedule
  - 2 will be vaccinated according to a 0,2,**6**-month schedule

- Group 2 double-blind IP allocation (n=20):
  - 16 subjects will receive 30 µg *Na*-APR-1 (M74) plus 5 µg GLA-AF delivered by IM injection in the deltoid muscle, with 30µg *Na*-GST-1 administered IM in the alternate arm.
    - 8 will be vaccinated according to a 0,2,**4**-month schedule
    - 8 will be vaccinated according to a 0,2,**6**-month schedule
  - 4 subjects will receive hepatitis B vaccine comparator:
    - 2 will be vaccinated according to a 0,2,**4**-month schedule
    - 2 will be vaccinated according to a 0,2,**6**-month schedule

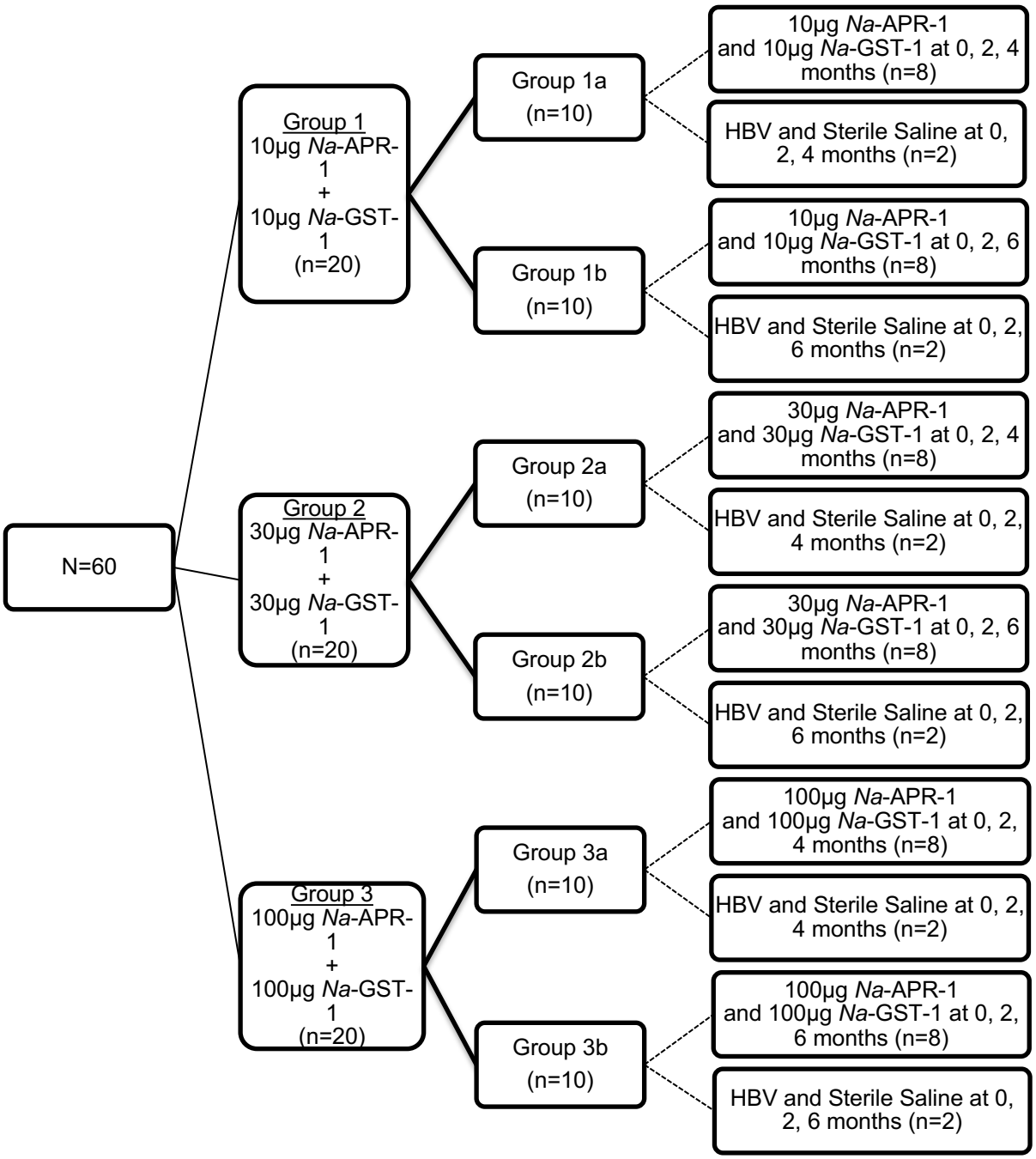
- Group 3 double-blind IP allocation (n=20):
  - 16 subjects will receive 100 µg *Na*-APR-1 (M74) plus 5 µg GLA-AF delivered by IM injection in the deltoid muscle, with 100 µg *Na*-GST-1 administered IM in the alternate arm.
    - 8 will be vaccinated according to a 0,2,**4**-month schedule
    - 8 will be vaccinated according to a 0,2,**6**-month schedule
  - 4 subjects will receive hepatitis B vaccine comparator:
    - 2 will be vaccinated according to a 0,2,**4**-month schedule
    - 2 will be vaccinated according to a 0,2,**6**-month schedule

Estimated Time to Complete Enrollment: 3 months

Schematic of Study Design:

Group Allocation: Open sequential enrollment ——————

Within Group IP Allocation: Double-blind randomized assignment - - - - - - - -



1 BACKGROUND INFORMATION AND SCIENTIFIC RATIONALE

1.1 Background Information

1.1.1 Hookworm Infection and Justification for Vaccine Development

There is an urgent need for new tools to control human hookworm infection and to reduce its burden of disease in developing countries. This is especially important for children and women of reproductive age who represent populations that are highly vulnerable to the effects of hookworm disease. Up to 65,000 deaths annually have been attributed to human hookworm infection (1). However, the mortality figures pale in comparison to global disease burden estimates that suggest that hookworm may account for the loss of up to 22 million Disability Adjusted Life Years annually (2), mainly due to the direct and indirect effects of acute and chronic anemia.

Human hookworm infection is a soil-transmitted helminth infection caused by the nematode parasites *Necator americanus* and *Ancylostoma duodenale*. It is one of the most common chronic infections of humans, affecting over 400 million people in the developing nations of the tropics (2). The largest number of cases occurs in impoverished rural areas of sub-Saharan Africa, Southeast Asia, China, and the tropical regions of the Americas. Approximately 3.2 billion people are at risk for hookworm infection in these areas. *N. americanus* is the most common hookworm worldwide, whereas *A. duodenale* is more geographically restricted (3).

Hookworm transmission occurs when skin comes into contact with infective third-stage larvae (L3) in fecally contaminated soil. The L3 have the ability to penetrate the skin, usually of the hands, feet, arms, buttocks and legs. The L3 invade human tissues and enter the gastrointestinal tract where they molt to the adult stage approximately 5-9 weeks following initial host entry. Adult hookworms are approximately 1 cm long parasites that cause host injury by attaching to the mucosa and submucosa of the small intestine to produce intestinal blood loss. There is a direct relationship between hookworm intensity (as determined by fecal egg counts) and host blood loss; typically the presence of between 40 and 160 adult hookworms in the intestine results in blood loss sufficient to cause anemia and malnutrition. The term “hookworm disease” refers primarily to the iron deficiency anemia and protein losses that occur in moderate and heavy infections (4). When host iron stores become depleted, there is a direct correlation between hookworm intensity and reduced host hemoglobin, serum ferritin, and protoporphyrin. Because of their low iron stores, children and women of reproductive age are the populations considered the most vulnerable to hookworm-associated blood loss (4-11).

In children, chronic hookworm infection and the resultant iron deficiency anemia have been shown to impair physical and intellectual development (3, 12, 13). Preschool children are particularly vulnerable to the effects of hookworm anemia and disease (8). In addition to its health impact on children, hookworm infection also affects adults. Unlike other soil-transmitted helminth infections such as ascariasis and trichuriasis, in which the highest intensity infections occur almost exclusively in school-aged children, it has been shown that high-intensity hookworm infections may also occur in adults (14-16).

The primary approach to hookworm control worldwide has been the frequent and periodic mass administration of benzimidazole anthelminthics to school-aged children living in high-prevalence areas. In 2001, the World Health Assembly adopted Resolution 54.19, which urges member states to provide regular anthelminthic treatment to high-risk groups with the target of regular treatment of at least 75% of all at-risk school-aged children. However, cure rates for a single dose of a benzimidazole are sub-optimal, particularly for mebendazole (17-20). These concerns have prompted interest in developing alternative tools for hookworm control (3, 21, 22). Vaccination to prevent the anemia associated with moderate and heavy intensity hookworm infection would alleviate the public health deficiencies of drug treatment alone.

The feasibility of developing a hookworm vaccine is based on the previous success of using live, irradiated hookworm larvae (L3 stage) as a vaccine for canine hookworm infection. This provided the experimental basis for the commercial development of a canine hookworm vaccine, which was marketed in the United States during the early 1970s. However, it is not realistic to develop a live L3 vaccine for humans due to multiple reasons including high production costs, challenging storage requirements, a short shelf life, and a lack of sterilizing immunity.

Alternatively, the strategy being pursued is to identify key hookworm proteins to which protective immune responses are directed in the animal models for this infection (namely the canine model) and to produce these as recombinant proteins that could then be used as vaccine antigens. This effort focused initially on identifying antigens expressed by the invading larvae (L3). In addition, a separate strategy has been to identify targets of the adult stage of the hookworm lifecycle; since hookworms attach onto the intestinal lumen and ingest host blood, antibodies could also be ingested that if directed against key hookworm proteins, would interfere with their function, ultimately resulting in the death or reduced fecundity of the worm.

1.1.2 Study Site

The study will be conducted in Lambaréné, Gabon, a city located in the Province of Moyen Ogooué, 240 km from Libreville, the capital of Gabon. There are approximately 35,000 people living in the urban municipal center of Lambaréné, with another 50,000 living in the surrounding rural areas. The area is located in the Central Africa rain forest setting at around 0°42'05S of latitude and 10°14'25E of longitude. The temperature average over the year is around 27°C. There are two annual rainy seasons (September to December and March to June) and two dry seasons (July to August and January to February). The city is bisected by the main river delta of the Ogooué. The majority of inhabitants in the urban area have tap water or access to supply (public wells) in the urban area, but in the rural areas, the water is supplied directly from small rivers. Latrines are common in the urbanised areas but not in rural areas. Income is generated mainly from farming, fishing and services. The urban part has two main hospitals and five pharmacies ("dispensaires"). Although rural areas are also served by several dispensaries, the quality of care varies. *Plasmodium falciparum* malaria, schistosomiasis, filariasis (*Loa loa* and *Mansonella perstans*), as well as soil-transmitted helminths including hookworm, *Ascaris lumbricoides, Strongiloides stercoralis* and *Trichuris trichiura* are endemic in the area.

Lambaréné has been chosen as a site for testing hookworm vaccines based on prevalence surveys conducted over the past few years, and the good working relationship that has been established between the research staff, the local health authorities, and the community. A recent survey using real time PCR yielded that 51% of 210 study participants assessed were found to

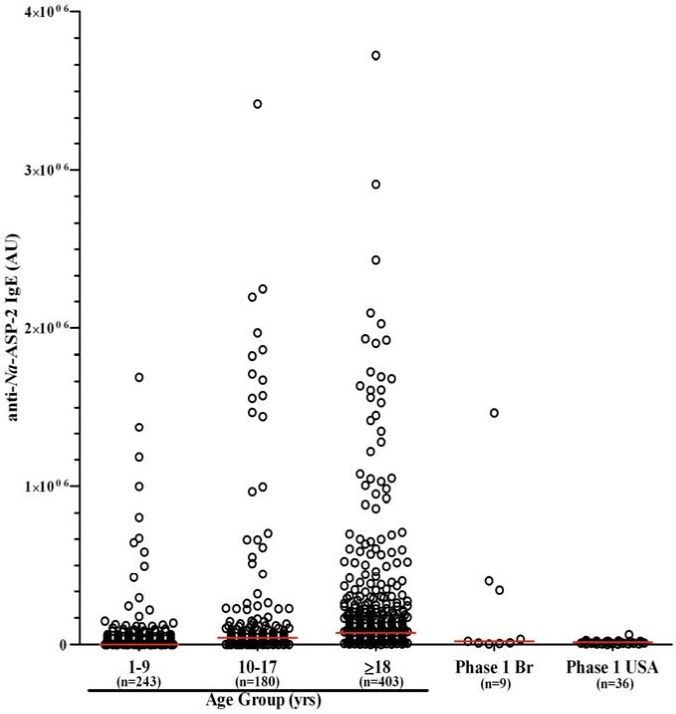
be positive for hookworm. The species identification yielded exclusively *Necator americanus*. (Adegnika et al working group)

1.1.3 Prior Clinical Experience with Hookworm Vaccines

The first hookworm vaccine to be tested in humans was the *Na*-ASP-2 (Ancylostoma Secreted Protein-2 of *N. americanus*) Hookworm Vaccine, consisting of recombinant *Na*-ASP-2 expressed in *Pichia pastoris* and adsorbed to aluminum hydroxide gel (Alhydrogel®). *Na*-ASP-2 is an excretory/secretory product produced by infective *N. americanus* larvae upon penetration of human skin. In animal models, vaccination with this recombinant antigen was shown to result in reduced worm burdens after challenge infection. Accordingly, a Phase 1 clinical trial of several different dose concentrations of the vaccine was conducted in healthy, hookworm-naïve adults living in the United States, which showed the formulation to be safe, well tolerated and immunogenic (23).

However, upon testing the vaccine in adults who had previously been infected with hookworm in Americaninhas, several volunteers in the lowest dose cohort to be vaccinated developed generalized urticaria within 2 hours of immunization (24). Due to these immediate-type hypersensitivity reactions, vaccinations in this study were halted. Subsequent investigations revealed that the volunteers who developed urticaria upon their first dose of *Na*-ASP-2 had elevated levels of baseline (i.e., pre-vaccination) IgE to the vaccine antigen. Subsequently, a sero-epidemiological survey was conducted in an endemic region of Brazil; this study revealed that even in young children, a significant proportion of individuals have detectable levels of IgE to this protein, likely due to previous infection with *N. americanus* (**Figure 1**). In addition, similar findings were demonstrated for other larval proteins that were being considered as vaccine candidates.

Figure 1: Anti-*Na*-ASP-2 IgE levels in residents of a hookworm-endemic region of northeastern Minas Gerais state, Brazil. Levels are shown for individuals of various age groups, in addition to 9 participants of the Phase 1 trial of *Na*-ASP-2 in Brazil and the participants of the Phase 1 trial of *Na*-ASP-2 that was conducted in hookworm-naïve adults in the United States (24).



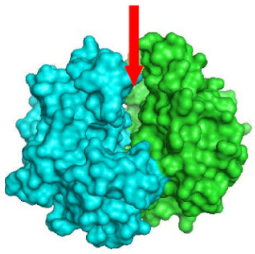
Due to these cumulative data, clinical development of the *Na*-ASP-2 and other larval-stage antigens as candidate vaccines was halted. Instead, the current strategy is to develop antigens expressed during the adult stage of the hookworm life cycle that play a role in digesting the host hemoglobin that is used by the worm as an energy source. These antigens do not induce specific IgE antibodies during natural infection, and hence have a low likelihood for inducing allergic reactions upon vaccination.

1.1.4 The *Na*-GST-1/Alhydrogel® Hookworm Vaccine

The nutritional and metabolic requirements of the adult hookworm living in the human intestine are dependent upon degradation of host hemoglobin that has been ingested by the worm. *N. americanus* hookworms depend on host hemoglobin for survival (25). Following hemolysis, adult hookworms use an ordered cascade of hemoglobinases to cleave hemoglobin into smaller molecules (25-30). Aspartic protease-1 of *N. americanus* (*Na*-APR-1) is responsible for initiating the proteolytic cascade in hookworms, as described below (**Section 1.1.7**). After hemoglobin digestion, the freed heme generates toxic oxygen radicals that can be bound and detoxified by molecules such as glutathione S-transferase-1 (GST-1) (31-33). GST-1 of *N. americanus* (*Na*-GST-1) is a critical enzyme that plays a role in parasite blood feeding; used as a vaccine, we hypothesize that the antigen will induce anti-enzyme neutralizing antibodies that will interfere with parasite blood-feeding and cause parasite death or reduce worm fecundity.

Na-GST-1 is a 24-kDa protein with peroxidase enzymatic activity that catalyzes the conjugation of reduced glutathione to a variety of electrophiles (31-33). This hookworm protein belongs to the Nu class of nematode GSTs, which also includes GSTs from the blood-feeding parasite of ruminants, *Haemonchus contortus*, and the rodent nematode *Heligomosoides polygyrus*. This class is characterized by diminished peroxidase activity relative to other classes of GSTs, but elevated binding capacity for heme and related products (31, 33-36). X-ray crystallography of *Na*-GST-1 demonstrates that the protein can form homodimers in solution, which create atypically large binding cavities accessible to a diversity of ligands, including heme (**Figure 2**) (33). *Na*-GST-1 binds heme at high affinity *in vitro* (31, 36). Because both heme and hematin contain oxidative iron, these molecules are potent generators of toxic reactive oxygen species that could potentially damage parasite macromolecules. *In vivo*, hookworm GSTs may therefore bind and detoxify the heme and hematin byproducts generated during the blood degradation process.

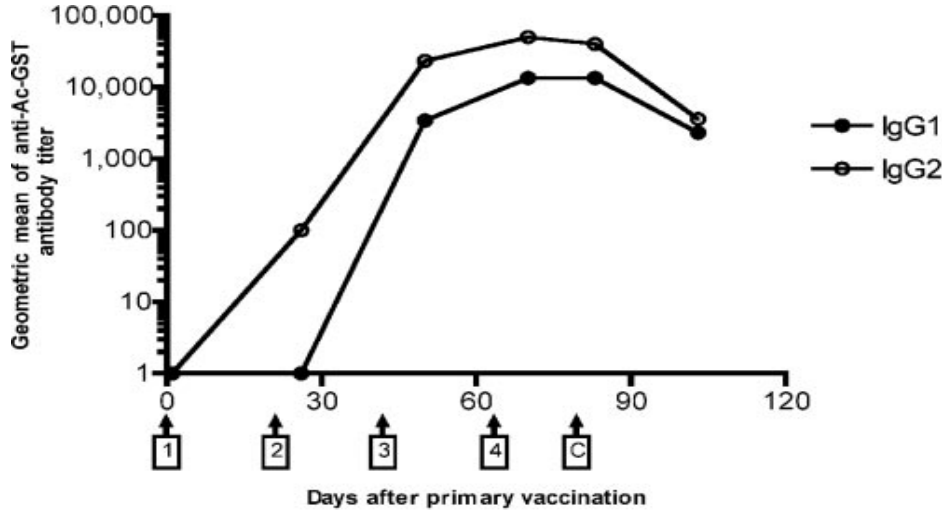
Figure 2: Three-dimensional surface plot of *Na*-GST-1. The path to the binding cavity is indicated by the red arrow (33).



Based on their putative role in hookworm blood feeding, both *Na*-GST-1 and its orthologue from the canine hookworm *Ancylostoma caninum* (*Ac*-GST-1) were tested as experimental vaccines in laboratory animal models of infection. In dogs, vaccination with recombinant *Ac*-GST-1 resulted in high levels of antigen-specific antibody (**Figure 3**); following challenge with *A. caninum* infective larvae, significantly lower host worm burdens and fecal egg counts were observed compared to control animals vaccinated only with adjuvant (31). In hamsters, vaccination with recombinant *Ac*-GST-1 also resulted in substantially lower worm burdens (51-54%) following heterologous challenge with *N. americanus* infective larvae compared to controls, as did vaccination with recombinant *Na*-GST-1 followed by homologous larval challenge (31, 32, 37). Because of these encouraging preclinical results, *Na*-GST-1 was manufactured according to current good manufacturing practices (cGMP) and formulated on Alhydrogel® in preparation for clinical trials.

Figure 3: Geometric mean titers of the IgG1 and IgG2 antibody responses of vaccinated dogs against recombinant *Ac*-GST-1 formulated with GlaxoSmithKline's

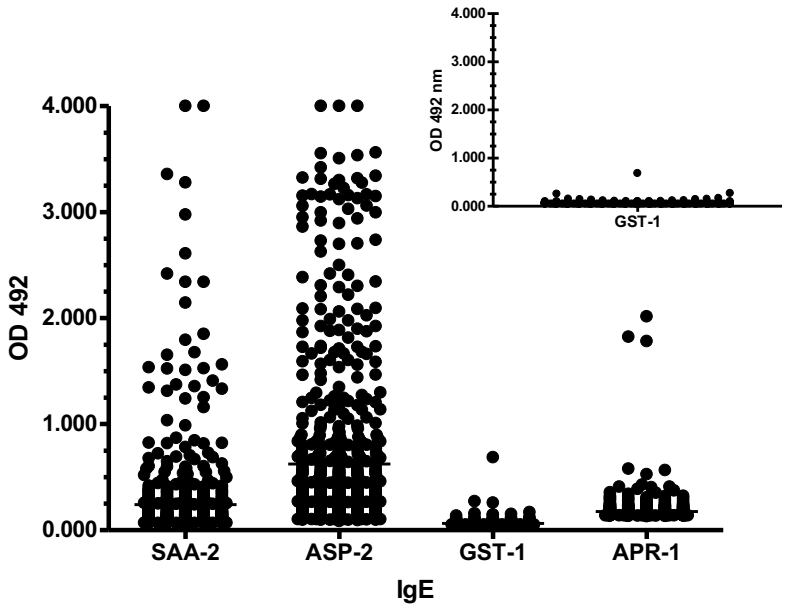
AS03 adjuvant. Vaccination time points (1, 2, 3, and 4) and challenge day (C) are marked with arrows (31).



Most importantly, extensive studies have been conducted to test for sensitization to the *Na*-GST-1 protein in individuals living in a hookworm endemic area who have been repeatedly exposed and infected with *N. americanus*. As shown in **Figure 4**, over 1000 individuals of all ages from a hookworm endemic area of Brazil have been tested for serum IgE antibodies to *Na*-GST-1 using an indirect ELISA. In addition, a subset of these serum samples stratified by age and infection status (n = 179) underwent confirmatory testing at the Johns Hopkins Dermatology, Allergy and Clinical Immunology Reference Laboratory (Baltimore, Maryland) using a custom ImmunoCAP assay. The ImmunoCAP method is considered the standard for measuring specific IgE to antigens in serum. This confirmatory testing demonstrated that none of the samples had *Na*-GST-1 IgE values above the clinical cut-off of 0.35 kU_A/L (**Figure 5**). Therefore, the likelihood of inducing immediate-type hypersensitivity reactions by vaccinating individuals living in hookworm-endemic areas with *Na*-GST-1 is low and likely not more than that associated with any new vaccine antigen entering clinical trials. The situation with *Na*-GST-1 is therefore very different from that of *Na*-ASP-2 in that repeated infection with hookworm does not induce an IgE response to the antigen, most likely due to the fact that it is a protein found in the digestive tract of adult hookworms and is therefore relatively hidden from the human immune system. This lack of antigen-specific IgE in people living in an area of high transmission has served as a major justification for advancing development of *Na*-GST-1 as a candidate vaccine antigen.

Figure 4: Anti-*Na*-GST-1 IgE levels in (A) adults and children and (B) young children aged 1-10 years (n=128) living in a hookworm-endemic area of Brazil. IgE levels (optical density at 492 nm) were measured by indirect ELISA.

A.



B.

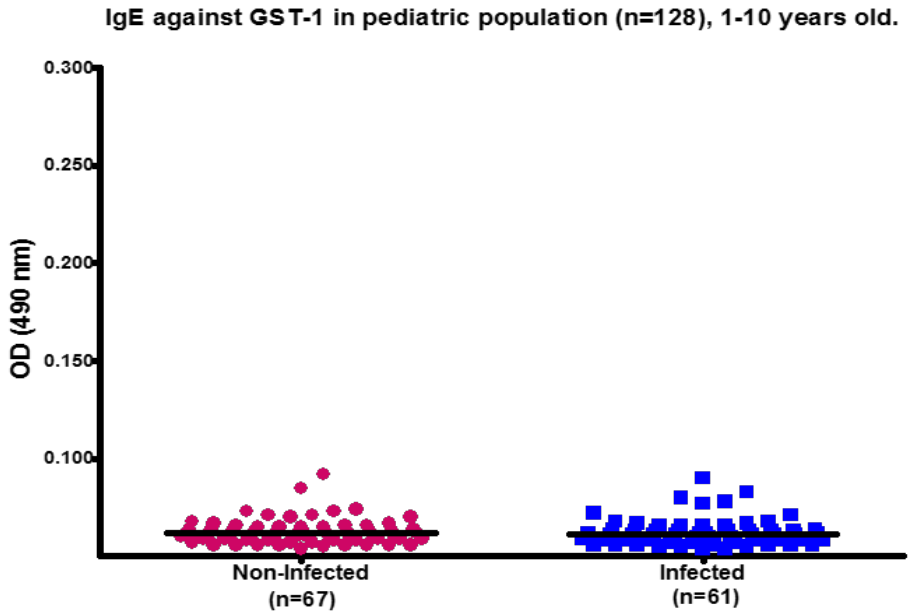
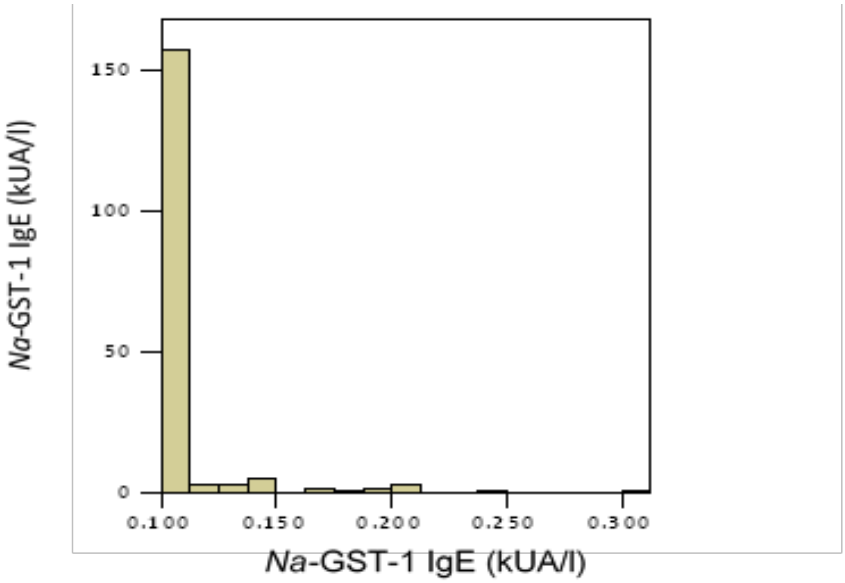


Figure 5: Anti-*Na*-GST-1 IgE levels in a subset of adults and children (n=179) living in a hookworm-endemic area of Brazil. IgE levels (kU_A/L) were measured by custom ImmunoCAP.



Na-GST-1 has been successfully manufactured and tested in the laboratory and in animals with both Alhydrogel® and Alhydrogel® plus GLA-AF. *Na*-GST-1 has been shown to be pure, potent, and stable in both of these two formulations.

The *Na*-GST-1 vaccine formulation to be tested in this study consists of the 24-kDa recombinant protein *Na*-GST-1, adsorbed to an adjuvant, Alhydrogel® (aluminum hydroxide suspension) with or without the addition of Gluco-Pyranosylphospho-Lipid A Aqueous Formulation (GLA-AF). The GLA-AF will be added to the Alhydrogel® formulation within 24 hours of immunization. The active ingredient is the recombinant *Na*-GST-1 protein that is derived by fermentation of *Pichia pastoris* yeast cells genetically engineered to express *Na*-GST-1.

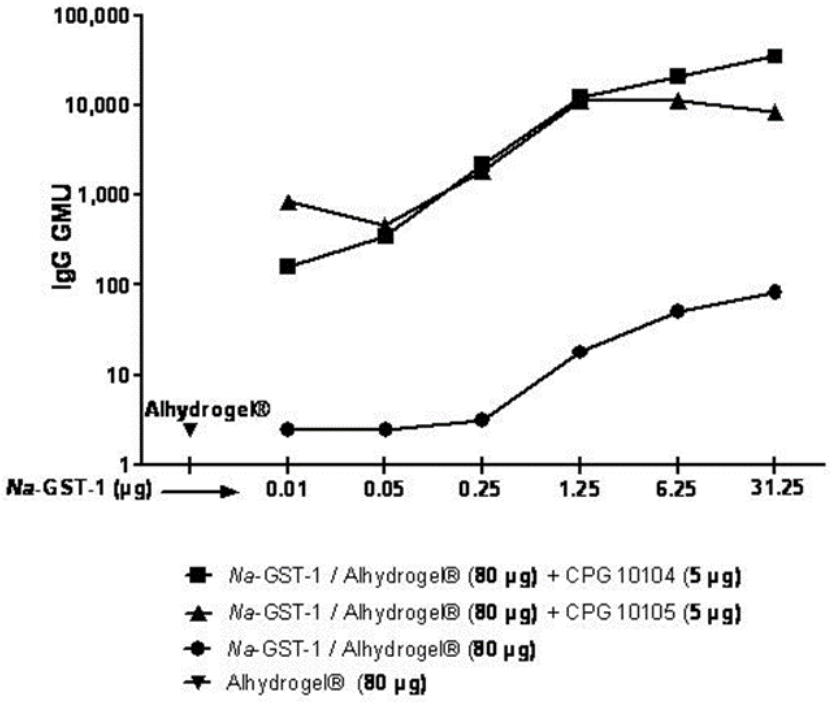
1.1.5 Immunogenicity Studies with *Na*-GST-1

Several preclinical animal studies have been conducted in both mice and rats to assess the immunogenicity of *Na*-GST-1 in combination with different adjuvants. First, a study conducted in Sprague-Dawley Rats demonstrated that the addition of an adjuvant to recombinant *Na*-GST-1 was necessary, since administration of the recombinant protein without an adjuvant resulted in minimal specific antibody responses.

A second study was conducted in BALB/c mice to assess the effect of co-administering CPG 10104 with recombinant *Na*-GST-1/Alhydrogel® (**Figure 6**). In this study, mice were vaccinated with *Na*-GST-1/Alhydrogel® at antigen doses ranging from of 0.01 to 31.25 µg *Na*-GST-1 with or without CPG 10104 (5 µg) or CPG 10105 (5 µg). CPG 10105 is a CpG oligodeoxynucleotide sequence that is similar to CPG 10104 but that is not being proposed to be tested in the study described in this protocol. Mice were vaccinated twice intramuscularly at a 3-week interval, with blood collected for anti-*Na*-GST-1 IgG ELISA two weeks after the second immunization. This study demonstrated a large, highly significant increase in IgG specific for *Na*-GST-1 in the group

administered *Na*-GST-1/Alhydrogel®/CPG 10104 compared to that administered only *Na*-GST-1/Alhydrogel® as shown in **Figure 6**.

Figure 6: Geometric mean anti-*Na*-GST-1 IgG antibody units 2 weeks after the 2nd Immunization of BALB/c mice with *Na*-GST-1/Alhydrogel® with or without co-administration of CPG 10104 or CPG 10105.



1.1.6 Clinical Experience with *Na*-GST-1/Alhydrogel® Hookworm Vaccine

The *Na*-GST-1/Alhydrogel® hookworm vaccine has been tested in adults in two Phase 1 clinical trials in humans in the United States (n=40), one in Brazil (n=102), and one in Gabon (n=32), the latter when coadministered with *Na*-APR-1/Alhydrogel®. To date, no significant reactogenicity or safety issues have been observed in any of these studies. In study SVI-11-01 in healthy, hookworm-naïve American adults conducted between 2012 and 2014 in Washington, DC, 40 volunteers received three vaccinations with up to 100 µg *Na*-GST-1/Alhydrogel® administered with or without up to 5 µg GLA-AF (NCT01385189). Mild to moderate injection site pain and tenderness were observed in a minority of study subjects; other common adverse events have included mild to moderate headache and nausea. The vaccine induced antigen-specific IgG antibody responses in a dose-dependent manner, although the addition of GLA-AF did not appreciably increase these responses over *Na*-GST-1/Alhydrogel® alone.

The trial in Brazil (SVI-10-01) tested *Na*-GST-1/Alhydrogel® in both hookworm-unexposed (n=36) and hookworm-exposed (n=66) healthy adults (NCT01261130). This study was initiated in Belo

Horizonte, a large urban area in the Brazilian state of Minas Gerais, in healthy, hookworm-unexposed adults (n=36) who received up to 100 µg *Na*-GST-1/Alhydrogel® with or without 2.5 µg GLA-AF. After no significant adverse events were observed in these volunteers, vaccinations were begun at the hookworm-endemic region of Americaninhas in Minas Gerais; this second part of the trial was a randomized, controlled, double-blind study in which subjects received up to 100 µg *Na*-GST-1/Alhydrogel® with or without 2.5 µg GLA-AF (n=60), or the recombinant hepatitis B vaccine (n=6). All volunteers in this study received three intramuscular injections at 0, 2, and 4 months and are being followed for 12 months after the final vaccination. In this study, *Na*-GST-1/Alhydrogel® (with or without GLA-AF) appeared to be safe and well tolerated in both hookworm-unexposed and hookworm-exposed adults. No vaccine-related SAEs have occurred and the vaccine appears to be well tolerated, with mild-to-moderate injection site pain and tenderness being the most common vaccine-related adverse events. Also, it is important to note that all volunteers who were screened for study SVI-10-01 were tested for IgE antibodies to *Na*-GST-1: none of these individuals in either Belo Horizonte or Americaninhas had detectable IgE antibodies to the vaccine antigen, as determined by ELISA. As in SVI-11-01, *Na*-GST-1/Alhydrogel® induced antigen-specific IgG responses in a dose-dependent fashion, but the addition of GLA-AF did not improve these responses. Due to the lack of improvement in antibody responses with the addition of GLA-AF to *Na*-GST-1/Alhydrogel®, the regulatory sponsor of the vaccine has recommended that GLA-AF should not be tested in combination with this product in future clinical trials.

1.1.7 The *Na*-APR-1 (M74)/Alhydrogel® Hookworm Vaccine

As described above, hookworms acquire their nutrition by ingesting blood, lysing the erythrocytes, and digesting the hemoglobin and serum proteins in the gut of the adult worm via a proteolytic cascade (28). Hookworm hemoglobinases have provided efficacy as recombinant subunit vaccines in animal models of hookworm disease, resulting in significant reductions in the intensity of infection and, most importantly, in protection against blood loss (29-31, 36). The *Na*-GST-1 and *Na*-APR-1 proteins are both components of the blood-feeding pathway of *N. americanus* and were selected for clinical development based on their protective efficacy in animal trials. *Na*-APR-1 is responsible for initiating the proteolytic cascade in *N. americanus* and catalytically active recombinant APR-1 has been found to confer protection in canine and hamster models of human hookworm disease (28, 30, 36). Vaccinated animals are thought to be protected as a result of induced antibodies that neutralize the catalytic activity of *Ac*-APR-1 in the gut of the worm (30).

To improve the stability of the recombinant enzyme, two aspartic acid residues were mutated to alanines to make *Na*-APR-$1_{mut,}$ which is the same as *Na*-APR-1 (M74), a catalytically inactivated mutant protein. Dogs vaccinated with recombinant *Na*-APR-1_{mut} were protected against blood loss and pathology; importantly, animals that were vaccinated with *Na*-APR-1_{mut} generated antibodies that neutralized the catalytic activity of wild type *Na*-APR-1 (29). Despite successfully producing large quantities of refolded *Na*-APR-1 and *Na*-APR-1_{mut} in *Escherichia coli*, the scalability of protein expression was hampered by issues of yield and aggregation (29). Therefore, as an alternative strategy, recombinant *Na*-APR-1 (M74) is derived by infiltration of *Agrobacterium tumefaciens* strain GV3101, genetically engineered to express *Na*-APR-1 (M74) in *Nicotiana benthamiana* tobacco plants.

The *Na*-APR-1 (M74)/Alhydrogel® vaccine is composed of the 42.18-kDa *Na*-APR-1 (M74) recombinant protein (0.1 mg/ml of the recombinant protein in 10 mM imidazole, 150 mM sodium chloride and 0.3% Empigen BB, with pH 7.4 ± 0.1), together with 0.8 mg/ml Alhydrogel®.

1.1.8 Studies of Human Immune Responses to *Na*-APR-1 in Endemic Areas

To assess presence of antibodies against *Na*-APR-1_{mut} in humans resident in *N. americanus* endemic areas, sera from 648 hookworm-infected individuals from Minas Gerais state, Brazil, were screened for anti-APR-1_{mut} antibody responses. Sixty-two percent showed detectable IgG responses (42% had IgG1, 0.6% IgG2, 15% IgG3, 53% IgG4) and only 0.6% had IgE antibodies to *Na*-APR-1_{mut} (Table 1). There was no association between the presence of antibody and current infection status (positive or negative), intensity of infection (EPG), or age.

Table 1: Anti-*Na*-APR-1_{mut} IgG, IgG subclasses (IgG1-4), IgE, and IgA Antibodies in Brazilian Individuals Living in a Hookworm-Endemic Area.

ELISA method	IgG1	IgG2	IgG3	IgG4	Total IgG	IgE	IgA
Seropositive [% (*n*)][a]	42 (270)	0.6 (4)	15 (97)	53 (343)	62 (401)	0.6 (4)	3 (22)
Levels in µg/ml[b]	0.195 ± 0.104	−*c*	0.123 ± 0.066	0.203 ± 0.136	0.592 ± 0.518	–	–

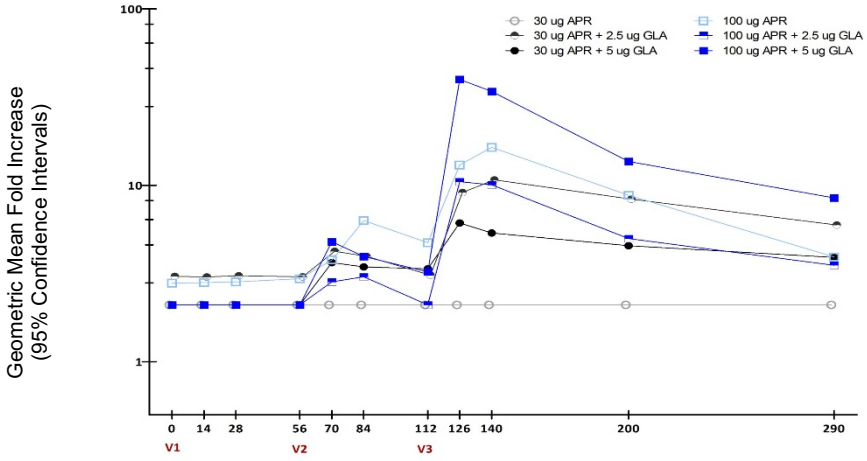
[a] Positivity as indicated by a reactivity threshold, which was determined from a frequency distribution from the upper 95% CI of the frequency distribution from log_{10} transformed OD values from a panel of sera from Brazilian volunteers not resident in an *N. americanus* transmission area (*n*=56) and a panel of control sera from U.S. volunteers (24).
[b] Values are means ± SD. Amounts were not calculated for samples in which <25 individuals were positive for the antibody.
[c] Heterologous interpolation of antibody levels to *Na*-APR-1_{mut} refers to a calibration method in which the standard curve uses a set of different reagents from those used to measure the analyte of interest; in this case, *Na*-APR-1_{mut}-specific antibody ELISA was simultaneously performed with purified human IgG, IgG1-4 and IgE bound to the solid phase followed by the appropriate 2nd antibody. The antibody values are determined by interpolating the OD of the sample values onto the heterologous standard curve.

The presence of IgG1 to *Na*-APR-1 in sera from humans resident in hookworm endemic areas resolves one of the most critical obstacles of gut antigens as targets for vaccines against blood-feeding helminths. Many consider antigens in the gut membrane of blood-feeding nematodes as "hidden," *i.e.*, not continuously presented to the immune system (34). The presence of circulating IgG1 in individuals in *Necator* endemic areas indicates that natural infection induces a host immune response to *Na*-APR-1 and that further immunization could augment this existing immune response. Importantly, the immune response to *Na*-APR-1 is neither of the IgG4 or IgE isotype as seen with other helminth antigens. This might be the result of expression of *Na*-APR-1 in the host gastrointestinal tract, compared to other organs through which hookworms pass, such as the skin or lung, and bias the immune response toward a T-helper type 2-cytokine profile. Based on the above studies, it is unlikely that *Na*-APR-1 (M74) will elicit an IgG4 or an IgE antibody response upon vaccination of individuals in high transmission areas, making this vaccine a highly viable hookworm vaccine candidate.

1.1.9 Clinical Experience with the *Na*-APR-1 (M74)/Alhydrogel® Hookworm Vaccine

The *Na*-APR-1 (M74)/Alhydrogel® Hookworm Vaccine has been tested in healthy, hookworm-unexposed adults in a Phase 1 clinical trial taking place in Washington, DC. In this study (SVI-12-01), 40 volunteers were vaccinated with either 30 µg or 100 µg *Na*-APR-1 (M74)/Alhydrogel® with or without 2.5 or 5 µg GLA-AF (NCT01717950). Three intramuscular vaccinations were administered to each volunteer according to a 0, 2, and 4-month schedule. The study was completed in July 2015. No vaccine-related SAEs were observed, and related AEs were limited mostly to mild-to-moderate injection site pain and tenderness. Immunogenicity results from this study demonstrated that *Na*-APR-1 (M74)/Alhydrogel® induces antigen-specific IgG responses in a dose-dependent manner, and that the addition of 5 µg GLA-AF noticeably improved these responses (**Figure 7**).

Figure 7. IgG responses in study SVI-12-01



1.1.1 Clinical Experience with *Na*-APR-1 (M74)/Alhydrogel® Co-Administered with *Na*-GST-1/Alhydrogel®

The current two candidate hookworm vaccines described above are being tested together in a Phase 1 clinical trial in Lambaréné, Gabon, at the same site as proposed in this protocol. In this study, HV001 (NCT02126462), 32 healthy, hookworm-exposed adults were vaccinated with 30 µg or 100 µg of both *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel® (n = 24) versus the comparator Hepatitis B Vaccine (n = 8). Both *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel® were mixed with 5 µg GLA-AF immediately prior to injection. Each study subject received three sets of intramuscular injections (one in each deltoid muscle) according to a 0, 1, and 6-month schedule. Vaccinations in this study were completed in September 2015. No vaccine-related SAEs were observed, and related AEs were limited mostly to mild-to-moderate injection site pain and tenderness, headache, nausea, and myalgia. Twelve subjects had 19 severe (grade 3) adverse events, of which one was an episode of high (severe) fever that was possibly related to vaccination while the rest were not considered related to vaccination. It should be noted that the study is yet to be unblinded and therefore it is not possible to state whether the AEs occurred in subjects who received the hookworm vaccines vs. the comparator hepatitis B vaccine.

1.1.2 Clinical Experience with Aluminum-Based Adjuvants

Several licensed vaccines contain aluminum-based adjuvants, including the recombinant hepatitis B vaccine, the tetanus toxoid vaccine, and the diphtheria-tetanus toxoids vaccine.(38, 39) For these aluminum hydroxide-adsorbed vaccines, local reactions such as pain, tenderness, and swelling are experienced in between 7.6% and 16.7% of volunteers in studies that included over 1,200 healthy adults. Fever is seen in 3.2% to 9.3%, headache in 4.1%, and other systemic symptoms such as fatigue, malaise, nausea, and diarrhea at lower frequencies. Urticaria has been reported in 0.1% of individuals vaccinated with the hepatitis B vaccine.

1.1.3 Clinical Experience with GLA-AF

Both *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel® are being tested in combination with the Toll-like Receptor 4 (TLR4) agonist, Gluco-Pyranosylphospho-Lipid A in Aqueous Formulation (GLA-AF, Infectious Diseases Research Institute [IDRI], Seattle, WA). GLA-AF contains a synthetic monophosphoryl lipid A (MPL) molecule that has TLR4 agonist activity. MPL is itself derived from the lipopolysaccharide (LPS) of *Salmonella minnesota*, a natural TLR4 agonist that is pyrogenic and can induce toxic shock. LPS, and more specifically, its lipid A component, has long been known for its strong adjuvant effects; however, its high toxicity has precluded its use in a vaccine formulation. Ribi et al showed that the monophosphorylated form of lipid A retains its adjuvant function and almost completely loses its endotoxin effects (40).

There have been many clinical trials involving thousands of subjects in which MPL or a derivative have been administered as vaccine adjuvants to adults and children, including vaccines for human papillomavirus, malaria (41-43), leishmaniasis (44), and hepatitis B (45). In general, these trials have demonstrated that administering MPL to humans is safe and well tolerated; when compared to formulations of vaccine that do not contain MPL, those adjuvanted with MPL may result in a minor increase in the incidence and/or severity of local injection site reactions. However, the addition of MPL also often results in a much improved specific antibody response to the vaccine antigen(s).

Of note, MPL is one of the components of the licensed Cervarix® vaccine (GlaxoSmithKline, Research Triangle Park, NC) for the prevention of cervical cancer due to human papillomavirus serotypes 16 and 18. The adjuvant for this vaccine consists of MPL adsorbed to aluminum hydroxide salt and is therefore similar to the combination of GLA-AF and Alhydrogel® that we propose testing in combination with *Na*-GST-1 and *Na*-APR-1 (M74) in the study described in this protocol. The Cervarix® vaccine has been shown to have a very favorable safety profile after having been tested in tens of thousands of healthy individuals (46, 47).

As mentioned previously, up to 5 µg of GLA-AF has been administered to a small number of human volunteers in combination with both *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel®. In addition, an oil-in-water emulsion of GLA (GLA-SE) has been used in combination with the Fluzone® trivalent killed influenza vaccine in a Phase 1 trial. In this study, doses up to 2.5 µg of GLA-SE were safe and well-tolerated and significantly enhanced influenza-specific antibody responses (48).

1.2 Rationale

1.2.1 Rationale for the Study

A product that combines *Na*-GST-1 and *Na*-APR-1 (M74) shows promise as an effective bivalent hookworm vaccine because vaccination of laboratory animals with recombinant *Na*-GST-1 or *Na*-APR-1 results in significant protection from challenge infections (29, 37). Therefore, vaccination of humans with recombinant *Na*-GST-1 or *Na*-APR-1 (M74) holds promise for inducing protection against this infection, particularly the moderate and heavy intensity infections that are associated with clinical sequelae such as intestinal blood loss and iron-deficiency anemia. The Sabin Vaccine Institute has sponsored a series of Phase 1 trials of the *Na*-GST-1 and *Na*-APR-1 (M74) candidate antigens in healthy adult volunteers from hookworm endemic areas (Brazil) and non-endemic areas (USA). Given the results of preclinical testing in laboratory animals, it is thought that a vaccine based on a single antigen will not be sufficient to provide adequate protection from disease (49). Instead, the goal is to develop a multi-component vaccine containing at least two recombinant proteins targeting different steps in the hookworm digestion of host hemoglobin. As such, a necessary step in clinical development is to test whether combining *Na*-GST-1 and *Na*-APR-1 (M74) results in an increased safety risk or reduced immunogenicity to either antigen, compared to when they are administered individually.

As a necessary first step, *Na*-GST-1 and *Na*-APR-1 (M74) were co-administered to healthy adults in Gabon, as described above in Section 1.1.1. However, given that the main target for an eventual vaccine will be children living in hookworm-endemic areas, pediatric Phase 1 trials are required to evaluate safety and immunogenicity in the target population prior to conducting larger-scale Phase 2 and 3 clinical trials.

1.2.2 Rationale for Doses and Dose Schedule to be Studied

Na-APR-1 (M74)/Alhydrogel® and *Na*-GST-1/Alhydrogel® (with or without GLA-AF) have been tested at the same field site as that proposed in the study described in this protocol, in healthy hookworm-exposed adults without any observed safety concerns. Therefore, in the study described in this protocol, these two vaccine products will be administered in escalating doses, to school-age children who live in an area where hookworm is endemic. Doses of 10, 30 and 100 µg of *Na*-APR-1 (M74) and *Na*-GST-1/Alhydrogel® were selected for evaluation based on preclinical studies and to maintain an equal fold-increase in the amount of antigen. The 10 µg dose was also selected as the lowest dose for clinical trials due to the difficulty in precisely dispensing lower volumes (both vaccine antigens have been formulated at a protein concentration of 100µg/ml). Selection of the maximum 100 µg doses of *Na*-GST-1 and *Na*-APR-1 (M74) is based on the safety and immunogenicity data from the Phase 1 studies of these antigens that have been conducted in adults, in which this was the maximum dose tested.

To date, up to 100 µg *Na*-APR-1 (M74)/Alhydrogel® administered with 5 µg GLA-AF has been very well tolerated in healthy adults, with no observed vaccine-related Serious Adverse Events and only mild to moderate local injection site pain and tenderness being the most commonly reported adverse events. However, the addition of GLA-AF to *Na*-GST-1/Alhydrogel did not significantly increase IgG levels to the *Na*-GST-1 antigen. Therefore, GLA-AF will not be tested further in combination with *Na*-GST-1/Alhydrogel.

For the study proposed in this clinical protocol, each child will be vaccinated three times, on Days 0, 56, and 112 or 180. On each vaccination day each child will receive two injections, one injection per arm. This vaccination schedule was selected to coordinate with the Expanded Program on Immunization as the human hookworm vaccine is intended to target children, including eventually infants. Two different vaccination schedules are being tested (i.e., 0, 2, 4 months *vs*. 0, 2, 6 months) to determine the optimal immunogenicity.

Local subcutaneous nodules, believed to be granulomatous reactions to aluminum hydroxide, have been observed with use of aluminum hydroxide-based adjuvants. Thus, most aluminum hydroxide-adsorbed vaccines are injected intramuscularly (IM) rather than subcutaneously, and intramuscular injection in the deltoid muscle will be used for administration of all investigational products in the study.

1.2.3 Clinical Development Plan for the Human Hookworm Vaccine

The target population for the human hookworm vaccine is children 10 years of age or younger living in hookworm endemic areas, since this is the age group that is most at risk of disease from this infection. Prior to conducting clinical trials in this age group, however, both *Na*-GST-1 and *Na*-APR-1 (M74) are currently being tested in healthy adults living in areas of high hookworm prevalence. Since co-administration of the two antigens in adults has not resulted in any significant safety concerns, testing is proceeding to Phase 1 testing in children living in hookworm endemic areas of Africa (Gabon, as described in this protocol) and Brazil. After safety and immunogenicity of the co-administered products is shown in children, testing of a co-formulated product that contains both *Na*-GST-1 and *Na*-APR-1 (M74) will be started, first in adults and then in children. The dose concentrations and components of the co-formulated product will be determined through the cumulative results of the study described in this protocol and the previous Phase 1 trials of these vaccine antigens.

The series of Phase 1 trials in adults and children described above will culminate in a large Phase 2 trial in which the co-formulated antigens will be administered to children living in endemic areas. The hookworm vaccine will be compared to a licensed comparator vaccine to evaluate the impact of mean fecal egg counts as well as a variety of clinical and parasitological endpoints. Assuming that an impact on infection is shown in the Phase 2 trial, that there are no new safety issues that arise due to co-formulation of the antigens, and that combining them into one product does not adversely affect the immunogenicity of either, this product will be tested in a pivotal multi-center Phase 3 trial in children. The primary endpoint of the Phase 3 trial will be the incidence of moderate and heavy hookworm infection (as determined by fecal egg counts) following administration of an anthelminthic and vaccination.

1.3 Potential Risks and Benefits

1.3.1 Potential Risks

Risks to subjects are those associated with venipuncture, intramuscular injection of the vaccines, possible reactions to the vaccines, and breach of confidentiality. Risks occasionally associated

with venipuncture include pain and bruising at the site of venipuncture, lightheadedness, and syncope (rarely). Intramuscular (IM) injection also may cause transient discomfort and fainting.

Possible local vaccine reactions include pain, swelling, erythema, induration, transient limitation of limb movement, lymphadenopathy, or pruritus at the injection site. Local subcutaneous nodules, believed to be granulomatous reactions to aluminum hydroxide, have been observed with use of aluminum hydroxide-based adjuvants, although these have not been observed with either *Na*-APR-1 (M74)/Alhydrogel® or *Na*-GST-1/Alhydrogel® in the currently ongoing trials of these products. Regardless, most aluminum hydroxide-adsorbed vaccines are injected intramuscularly rather than subcutaneously. Systemic reactions such as fever, headache, malaise, myalgia, and joint pain, may also possibly occur. Immediate hypersensitivity reactions including urticaria, anaphylaxis, or other IgE-mediated responses are possible as with any vaccine. As with any investigational vaccine, there is a theoretical possibility of risks about which we have no present knowledge. Subjects will be informed of any such risks should further data become available.

Phase 1 trials of *Na*-APR-1 (M74)/Alhydrogel® plus GLA-AF have demonstrated similar reactogenicity and safety to the antigen administered without GLA-AF. However, in a study of an influenza vaccine administered with GLA-SE (a formulation similar to GLA-AF), the frequency and intensity of local reactions were greater than when the vaccine was administered without GLA-SE (50).

1.3.2 Precautions Taken to Minimize Risks

In order to minimize the risk to subjects, all children will be monitored closely during their participation in this study. The study vaccines and GLA-AF have been produced according to current Good Manufacturing Practices (GMP) and will be administered by experienced investigators with drugs and equipment available for the treatment of anaphylaxis and other potential adverse reactions. All vaccine doses will be given by intramuscular injection to minimize injection site reactions such as pain.

Maintenance of Confidentiality
Participants will be asked to provide personal health information. All attempts will be made to keep this information confidential within the limits of the law. However, there remains the unlikely risk that unauthorized persons will see the subjects' personal health information. All records will be kept in a locked file cabinet or maintained in a locked room at the study site. Electronic files will be password protected. Only people who are involved in the conduct, oversight, monitoring, or auditing of this study will be allowed access to the personal health information that is collected. Any publications from this study will not use information that will identify subjects by name.

1.3.3 Known Potential Benefits

Children who are enrolled in the study may not receive any direct benefit from participation. It is hoped that information gained in this study will contribute to the development of a safe and effective hookworm vaccine. All children will undergo a stool examination for ova and parasites at screening and on Day 140 and Day 380 study visits. Those found to have a helminth infection

at either of these study visits will be offered appropriate treatment at the conclusion of the study, free-of-charge. Free medical treatment will be provided to all vaccinated subjects during the active immunization phase and the follow-up period. If the investigators judge that a subject requires hospitalization, transportation will be arranged and the medical management of the subject will be monitored by a study physician and the local Independent Safety Monitor. Medical care for ailments not related to vaccination will not extend beyond the study period, but will be referred to the nearest government-run health clinic. Medical care for ailments related to vaccination will extend at least until the condition has resolved or stabilized (if a chronic condition).

2 OBJECTIVES

2.1 Study Objectives

Primary:

Safety

1. To evaluate the safety and reactogenicity of three different dose concentrations of *Na*-APR-1 (M74)/Alhydrogel® co-administered with *Na*-GST-1/Alhydrogel® in healthy Gabonese children.

Secondary:

Immunogenicity

2. To determine the doses of co-administered *Na*-APR-1 (M74) and *Na*-GST-1 that result in the highest levels of IgG against these vaccine antigens approximately 14 days after the third vaccination.

Tertiary:

Immunogenicity

1. To assess and compare the duration of antibody responses to *Na*-GST-1 and *Na*-APR-1 (M74).
2. To assess the level of IgG1-4 subclass responses to *Na*-APR-1 (M74) and *Na*-GST-1.

Exploratory:

Immunogenicity

1. To assess the cellular immune responses to the *Na*-GST-1 and *Na*-APR-1 (M74) antigens following immunization.
2. To assess the impact of co-administered *Na*-GST-1 and *Na*-APR-1 (M74) on the production of memory B cells specific for each antigen.
3. To assess the production of memory B cells specific for each vaccine antigen compared to metabolomic changes before and after vaccination.
4. To characterize the metabolite composition in urine and serum during the course of immunization and assess the changes on metabolite profiles related to each dose of co-administration of Na-GST-1 and Na-APR-1 (M74).

2.2 Study Outcome Measures

2.2.1 Primary Outcome Measures

Safety

The following parameters will be evaluated for each dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. Frequency of solicited injection site and systemic reactogenicity, graded by severity, on the day of each study vaccination through 14 days after each study vaccination.
2. Frequency of study vaccine-related serious adverse events from the time of the first study vaccination through approximately 9 months after the last study vaccination.
3. Frequency of clinical safety laboratory adverse events.
4. Frequency of unsolicited adverse events, graded by severity, from the time of each study vaccination through approximately 1 month after each study vaccination.
5. Frequency of new-onset chronic medical conditions through approximately 9 months after the third study vaccination.
6. Frequency of Adverse Events of Special Interest through approximately 9 months after the third study vaccination.

2.2.2 Secondary Outcome Measure

Immunogenicity

The following parameter will be evaluated for each dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. The IgG level as measured by a qualified indirect enzyme-linked immunosorbent assay (ELISA) for each vaccine antigen approximately 14 days after the third vaccination.

2.2.3 Tertiary Outcome Measures

Immunogenicity

The following parameters will be evaluated for each dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. The IgG antibody response as measured by a qualified indirect ELISA at approximately 7, 14, and 28 days after each vaccination, and approximately 3 and 6 months after the third dose.
2. The IgG subclass levels (IgG1, IgG3, and IgG4) against the vaccine antigens by indirect ELISA approximately 14 days after the third vaccination.

3. The IgG1-4 subclass levels as measured by indirect ELISA on each day of vaccination, approximately 28 days later, and approximately 3 and 6 months after the final vaccination.

2.2.4 Exploratory Outcome Measures

The following parameters will be evaluated for each dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. Cellular immune response will be measured by a qualified indirect ELISA at approximately on days of vaccination, and then 7, 14, and 28 days after each vaccination, and approximately 3 and 6 months after the third dose.
2. Metabolomic profiles before vaccination, on days of vaccination, approximately 7 and 28 days following each vaccination, and 6 months after the third dose.

3 STUDY DESIGN

The study will be conducted as a randomized, placebo-controlled, double-blind Phase 1 dose-escalating clinical trial in healthy children between the ages of 6 and 10 years, inclusive, living in the hookworm-endemic area of Lambaréné, Gabon. The study is designed to evaluate the safety, reactogenicity, and immunogenicity of *Na*-GST-1/Alhydrogel® co-administered with *Na*-APR-1 (M74)/Alhydrogel®, as compared to the hepatitis B vaccine, in this age group.

Prior to beginning study-related activities, the consent and cooperation of representative community members will be sought, after which parents of potential pediatric participants will be invited to learn about the study and consent to the participation of their child. After a parent or legal representative provides written informed consent for the child's participation and passes the informed consent comprehension questionnaire, the child will undergo eligibility screening, including a complete medical history, physical examination, hematology testing, liver and renal function testing, HIV antibody testing, Hepatitis B and C serology, and urinalysis. In addition, a fecal examination will be performed for ova and parasites, and urine will be examined for *S. haematobium* ova. All clinically significant abnormalities will be reviewed with parents/legal guardians and referral of the child for follow-up care will be provided. After screening, those children determined to be eligible, based on the inclusion and exclusion criteria, will be invited to participate in the study.

Sixty children will be progressively enrolled into 1 of 3 Groups over a projected 3-month enrollment period, with each child followed for 9 months after the final injection (i.e., until Study Day 380 for those receiving vaccinations according to a 0,2,4-month schedule and until Study Day 448 for those receiving vaccinations according to a 0,2,6-month schedule). Group enrollment will be done in an open sequential fashion, whereas within each Group, investigational product (IP) assignment and vaccination will be done in a randomized double-blind fashion.

60 subjects will be enrolled into 3 groups of 20:

The first 20 subjects will be recruited and enrolled into Group 1:

- Group 1 double-blind IP allocation (n=20):
    - 16 subjects will receive 10µg *Na*-APR-1 (M74) plus 5µg GLA-AF delivered by IM injection in the deltoid muscle, with 10µg *Na*-GST-1 administered IM in the alternate arm.
        - 8 will be vaccinated according to a 0,2,**4**-month schedule
        - 8 will be vaccinated according to a 0,2,**6**-month schedule
    - 4 subjects will receive hepatitis B vaccine comparator:
        - 2 will be vaccinated according to a 0,2,**4**-month schedule
        - 2 will be vaccinated according to a 0,2,**6**-month schedule

- Group 2 double-blind IP allocation (n=20):
    - 16 subjects will receive 30µg *Na*-APR-1 (M74) plus 5µg GLA-AF delivered by IM injection in the deltoid muscle, with 30µg *Na*-GST-1 administered IM in the alternate arm.
        - 8 will be vaccinated according to a 0,2,**4**-month schedule

        - 8 will be vaccinated according to a 0,2,**6**-month schedule
    - 4 subjects will receive hepatitis B vaccine comparator:
        - 2 will be vaccinated according to a 0,2,**4**-month schedule
        - 2 will be vaccinated according to a 0,2,**6**-month schedule

- Group 3 double-blind IP allocation (n=20):
    - 16 subjects will receive 100µg *Na*-APR-1 (M74) plus 5µg GLA-AF delivered by IM injection in the deltoid muscle, with 100µg *Na*-GST-1 administered IM in the alternate arm.
        - 8 will be vaccinated according to a 0,2,**4**-month schedule
        - 8 will be vaccinated according to a 0,2,**6**-month schedule
    - 4 subjects will receive hepatitis B vaccine comparator:
        - 2 will be vaccinated according to a 0,2,**4**-month schedule
        - 2 will be vaccinated according to a 0,2,**6**-month schedule

As with other aluminum hydroxide-adsorbed vaccines, hypersensitivity reactions would be expected to occur within the first 24 hours after receipt of either of the two vaccines, and other severe local or systemic reactions within 72 hours of vaccination. Children will therefore be observed for immediate reactions following each vaccination for at least 1 hour, and will have a clinical assessment either in their home or at the study clinic on Days 1, 3, 7, and 14 following each vaccination. See **Appendix A** for the schedule of clinical and laboratory evaluations.

In order to review safety data at each dose of *Na*-APR-1 (M74)/Alhydrogel® prior to testing it in combination with the point-of injection addition of GLA-AF, and to review safety data at each dose of *Na*-APR-1 (M74)/Alhydrogel® prior to dose escalation, Groups will be enrolled and vaccinated in a staggered fashion. Conference calls between the investigators and the Safety Monitoring Committee (SMC) will be scheduled within the week prior to beginning vaccinations in Groups 2 and 3. A cumulative safety report will be submitted to the SMC before beginning vaccinations in these Groups that will include safety data from at least the first 14 days after vaccination of all subjects in the preceding Group. Written approval (via fax or email) to proceed to vaccinations of Groups 2 and 3 must be obtained from the SMC.

The SMC and Independent Medical Monitor will have access to the randomization code for the study, as they may wish to review the data in an unblinded fashion should significant safety questions arise prior to the final unblinding. The trial will not proceed to the next dose Group if any of the stopping criteria listed in **Section 8.5** are met or, in the clinical judgment of the SMC or the Independent Medical Monitor the next higher dose would pose an unacceptable safety risk to the subjects.

4 STUDY ENROLLMENT AND WITHDRAWAL

Only children who meet all of the inclusion and none of the exclusion criteria will be eligible for enrollment into this study. No exemptions will be granted.

A total of 60 children will be enrolled. The study population will be enrolled from in and around Lambaréné.

Parents/legal guardians who agree to the participation of their child will first provide written informed consent and take a true/false comprehension questionnaire. The questionnaire will be administered orally in the case of illiterate volunteers, and for both written and oral questionnaires study staff will use incorrect answers to identify aspects of the study that require clarification and focus on those areas of the informed consent form for further review with the volunteer. All questionnaire questions must be answered correctly, and the informed consent form signed, prior to study screening and enrollment. Parents or legal guardians who are unable to read will place an imprint of their finger in the place of a signature; in addition, an independent witness, who is not a member of the study team, will sign the consent form to attest that the informed consent form was read to the parent/legal guardian, their questions were answered, and the parent/legal guardian answered all comprehension questions correctly. The original signed informed consent form for each parent/guardian will be maintained as part of that child's study records. A copy of the informed consent form will be provided to every parent/guardian.

Screening can occur up to 90 days prior to enrollment; enrollment/randomization, and administration of first dose of study products will occur on the same day.

4.1 Subject Inclusion Criteria

1. Males or females between 6 and 10 years, inclusive, who are long-term residents of the study area.
2. Good general health as determined by means of the screening procedure.
3. Assumed availability for the duration of the trial (up to 15 months).
4. Willingness of parent or legal guardian for child to participate in the study as evidenced by signing the informed consent document in combination with the child assent form.
5. Negative for hookworm during screening, or if found to be infected with hookworm, has completed a course of three doses of albendazole.

4.2 Subject Exclusion Criteria

1. Inability of parent/legal guardian to correctly answer all questions on the informed consent comprehension questionnaire.
2. Evidence of clinically significant neurologic, cardiac, pulmonary, hepatic, rheumatologic, autoimmune, diabetes, or renal disease by history, physical examination, and/or laboratory studies.
3. Known or suspected immunodeficiency.
4. Laboratory evidence of liver disease (alanine aminotransferase [ALT] greater than 1.25-times the upper reference limit).

5. Laboratory evidence of renal disease (serum creatinine greater than 1.25-times the upper reference limit, or more than 1+ protein, or more than trace blood on urine dipstick testing with the exception of greater than trace blood detected in females during menses).
6. Laboratory evidence of hematologic disease (absolute leukocyte count <4500/mm^3; absolute leukocyte count >13.0 x $10^3/mm^3$; hemoglobin <9.5 g/dl; or, platelet count <140,000/mm^3).
7. Other condition that in the opinion of the investigator could jeopardize the safety or rights of a child participating in the trial or would render the child unable to comply with the protocol.
8. Participation in another investigational vaccine or drug trial within 30 days of starting this study or for the duration of the study.
9. History of a severe allergic reaction or anaphylaxis.
10. Severe asthma as defined by the need for daily use of inhalers or emergency room/clinic visit or hospitalization within 6 months of the child's planned first vaccination in the study.
11. Positive for HCV.
12. Positive for HBsAg.
13. Positive for HIV infection.
14. Use of corticosteroids (excluding topical or nasal) or immunosuppressive drugs within 30 days of starting this study or expect to use for the duration of the study.
15. Receipt of a live vaccine within past 4 weeks or a killed vaccine within past 2 weeks prior to entry into the study.
16. History of a surgical splenectomy.
17. Receipt of blood products within the 6 months prior to entry into the study.
18. Previous receipt of a primary series (three doses according to a 0, 1, and 6 -12 month schedule) of the hepatitis B vaccine.

4.3 Vaccine Assignment Procedures

4.3.1 Randomization Procedures

Parents/guardians of eligible children will be asked to bring them to the clinic on the scheduled day of enrollment into the study (Day 0). If required, transportation to the clinic will be provided free-of-charge. One or 2 additional eligible children above the number to be enrolled into a given Group may be scheduled as Day 0 visit alternates, if possible. After undergoing a clinical interview and physical examination to ensure that they remain eligible for participation in the study, that they have had blood collected for safety clinical laboratory and baseline immunogenicity assessments, children will be randomized and enrolled in the order that they present for vaccination.

Within each group, randomization will be done through use of a randomization code, furnished to the study vaccine manager by the data management center (AIGHD). Access to the randomization list will be exclusively limited to the study vaccine manager and assistant. Between vaccination days, the randomization list will be stored in a locked cabinet. The study vaccine manager and assistant will be unblinded, but will not be involved in further evaluation of study subjects or assessment of adverse events. Children will not be considered enrolled in the study until they have received their first dose of vaccine. In the event that a child is randomized but not enrolled on the day of first vaccination, they will be replaced with an eligible alternate. Enrolled children that leave the study for any reason following first vaccination will not be replaced. Any alternates not vaccinated will be invited to participate as members of the next Group. Any Group

3 alternates not vaccinated, and therefore not enrolled in the study, will be offered vaccination with a non-study, licensed vaccine such as the tetanus toxoid or influenza vaccine.

4.3.2 Allocation of vaccination schedule and vaccine randomization

Within each cohort the first 10 participants will receive the 0,2,4-month vaccination schedule and the last 10 will receive the 0,2,6-month schedule. The participants will be randomized to either the experimental group, receiving Na-APR-1(M74) plus 5 µg GLA-AF co-administered with Na-GST-1, or randomized to the control group who will receive HBV co-administered with sterile saline. In addition, there will be a randomization to assign left/right arm vaccination for the first injection, the arms will be alternated for the second and third sets of vaccinations. The Randomization list will thus only be used on Day 0 for a particular participant.

The vaccination manager assigns the Study ID when the vaccines are being prepared for that particular participant. The Study ID is written onto the syringes and then the vaccinator writes the Study ID onto the CRF.

4.3.3 Masking Procedures

Due to the staggered, dose-escalation design of the trial, it will not be possible to blind to *Na*-APR-1 (M74)/Alhydrogel® or *Na*-GST-1/Alhydrogel® dose assignment. That is, those subjects enrolled into Groups 1 and 2 will receive either 10µg *Na*-APR-1 (M74)/Alhydrogel® administered to one arm and 10µg *Na*-GST-1/Alhydrogel® administered to the alternate arm, or the hepatitis B vaccine in one arm and sterile saline administered to the alternate arm. Subjects enrolled into Groups 3 and 4 will receive either 30µg *Na*-APR-1 (M74)/Alhydrogel® administered to one arm and 30µg *Na*-GST-1/Alhydrogel® administered to the alternate arm, or the hepatitis B vaccine in one arm and sterile saline administered to the alternate arm, and so on.

Investigators and subjects will be blinded to the within-Group random allocation to receive co-administered *Na*-APR-1 (M74)/Alhydrogel® and *Na*-GST-1/Alhydrogel® or co-administered hepatitis B vaccine and sterile saline until all subjects have completed their Day 208 visit, the safety and secondary immunogenicity outcomes (i.e., anti-*Na*-GST-1 and anti-*Na*-APR-1 [M74] IgG antibody results by ELISA) have been monitored and entered into the database, and the database has been “soft-locked” for interim analysis. Procedures to maintain blinding include administering two injections to all subjects at each vaccination point. For this, sterile saline will be co-administered to those subjects randomized to receive the hepatitis B vaccine.

The study vaccine manager and his assistant will prepare all investigational product doses (vaccine or sterile saline placebo) in a separate room, and will hand filled syringes to the vaccinator(s). Since the 10 and 30µg doses of the *Na*-APR-1 (M74) and *Na*-GST-1 formulations are of different volumes than that of hepatitis B vaccine, the contents of all syringes will be disguised using opaque tape. As a further precaution, the vaccinator(s) will not be involved in assessments of reactogenicity or adverse events.

Investigators will be unblinded after all subjects have had their study Day 208 visit (i.e., 1 month after the final vaccination for those children randomized to receive vaccines on the 0, 2, and 6-month schedule), the safety and secondary immunogenicity outcomes (i.e., anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) IgG antibody results by ELISA) have been monitored and entered into the database, and the database has been "soft-locked" for interim analysis. After this point, the study will be single-blinded (i.e., subjects will remain blinded to what products they received). The principal justification for unblinding the investigators before the final study visit has been completed is that the safety and immunogenicity data acquired during the vaccination phase of the study will be crucial in guiding the human hookworm vaccine clinical development plan, and we feel that to make an informed decision as to whether or not to proceed to pediatric trials, we must first assess the unblinded data in a timely fashion.

4.3.4 Reasons for Withdrawal and Discontinuation of Vaccinations

Parents/guardians will be free to withdraw their child from the study at any time and for any reason, without penalty. Children who have received vaccine, regardless of the number of doses received, or who developed an AE or SAE will be strongly encouraged to remain in the study to be followed for safety purposes.

A child may withdraw or be withdrawn from the study for any of the following reasons:

- Withdrawal of consent of the parent or legal guardian.
- Withdrawal of assent of the child.
- As deemed necessary by the Principal Investigator or appropriate co-investigator for noncompliance or other reasons.
- Subject lost to follow-up.
- Early termination of the study.

The second or third study vaccination may not be administered to a child if any of the following criteria are met (although the child will remain in the study for safety follow-up visits):

- Medical condition for which continued participation, in the opinion of the Principal Investigator or appropriate co-investigator, would pose a risk to the child or would be likely to confound interpretation of the results.
- Presence of signs or symptoms that could confound or confuse assessment of study vaccine reactogenicity. For children with injection site or systemic signs or symptoms, or with an acute illness, including a tympanic temperature greater than 38.0°C, the study vaccination should/may be postponed/deferred until signs, symptoms, or acute illness have resolved and if within the acceptable protocol-specified window for that visit. If outside this window, the Sponsor must first approve the second or third study vaccination and the documentation of approval should be filed in the child's chart.

- Any unresolved or continuing solicited or unsolicited Grade 3 adverse event. An unresolved or continuing Grade 1 or Grade 2 adverse event is permissible unless, in the opinion of the Principal Investigator or appropriate co-investigator, it would render study vaccination unsafe or interfere with the evaluation of responses.

- Grade 3 clinical safety laboratory value that does not decrease to Grade 2 or less prior to the second or third study vaccination. Any clinical safety laboratory parameter may be re-evaluated only once at the clinical laboratory prior to the second or third study vaccination. If the clinical safety laboratory value decreases to Grade 2 or less, the child may receive the second or third study vaccination. The second or third study vaccination should be scheduled to occur within the acceptable protocol-specified window for that visit. If outside this window, the Sponsor must first approve the study vaccination and the documentation of approval should be filed in the child's chart.

- Severe or sustained reaction or disability related to the first study vaccination.

- New onset of illness or condition that meets exclusion criteria.

- Child no longer meets eligibility criteria.

- As deemed necessary by the Principal Investigator or appropriate co-investigator for noncompliance or other reasons.

- Refusal of further study vaccination by the parent/guardian.

- Withdrawal of consent of the parent or legal guardian.

- Child lost to follow-up.

- Early termination of the study.

- New information becomes available that makes further vaccinations unsafe.

4.3.5 Handling of Withdrawals

The primary reason for withdrawal from the study will be recorded on the appropriate data collection form. Parents/guardians will be encouraged to have their child complete the Early Termination Visit. The Early Termination Visit procedures are listed in **Section 7.5**. Although children are free to withdraw at any time or may be withdrawn by the Principal Investigator or appropriate co-investigator at any time, those who receive at least one dose of study vaccines will be encouraged to remain in the study for follow-up safety assessments and collection of venous blood samples for immunogenicity testing. Every attempt will be made to follow all adverse events, including solicited injection site and systemic reactions, serious adverse events, and new-onset chronic medical conditions ongoing at the time of early withdrawal to resolution.

In the case of children who fail to appear for a follow-up safety assessment, extensive efforts (i.e., three documented contact attempts via in-person visits to the child's home, made on separate

occasions) will be made to locate or recall them, or at least to determine their health status. These efforts will be documented in the child's records.

4.3.6 Termination of Study

If a dose of vaccine is considered significantly reactogenic (see Section 8.5) dose escalation and/or additional vaccinations will be suspended until reviewed by the Independent Medical Monitor, SMC, and study sponsor. Any recommendation of the Independent Medical Monitor or SMC to resume or suspend further injections (either for an individual child or an entire dose Group) will be communicated in writing to the sponsor and Principal Investigator. All communications from the SMC will subsequently be forwarded by the investigators to the IRB.

5 STUDY INTERVENTIONS/INVESTIGATIONAL PRODUCTS

Investigators will receive the current versions of the Clinical Investigator's Brochures for *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel®, which comprehensively describe all available preclinical and human experience with the experimental vaccines. If relevant new information becomes available during the course of the trial, the investigators will receive the revised Investigator's Brochure(s).

5.1 Study Product Description

5.1.1 Acquisition

Vials of *Na*-GST-1/Alhydrogel®, *Na*-APR-1 (M74)/Alhydrogel®, GLA-AF, ENGERIX hepatitis B vaccine, and sterile saline for injection for this study will be supplied to the study site. *Na*-GST-1/Alhydrogel®, *Na*-APR-1 (M74)/Alhydrogel®, and GLA-AF will be transported to the study site at 0.5°C to 10°C; temperature recording devices will accompany the vaccines at all times during transport to ensure temperature limits have not been violated. ENGERIX hepatitis B vaccine and sterile saline will be purchased at the study site.

5.1.2 Formulation, Packaging, and Labeling

5.1.2.1 *Na*-APR-1 (M74)/Alhydrogel®

Na-APR-1 (M74)/Alhydrogel® is supplied as a sterile milky-white suspension (when shaken slightly). Each 2.0 ml vial contains a 0.1 mg/ml suspension of *Na*-APR-1 (M74) adsorbed to 0.8 mg/mL of Alhydrogel® in a solution containing 10 mM imidazole, 150 mM sodium chloride and 0.3% Empigen BB, with pH 7.4 ± 0.1. The maximum dose that will be administered is 100 µg of *Na*-APR-1 (M74), or 1.0 ml of the final drug product. This volume contains the equivalent of approximately 400 µg aluminum. Lower doses of *Na*-APR-1 (M74) are delivered by injecting smaller volumes of the 0.1 mg/ml suspension: for example, 0.3 ml will be injected to deliver 30 µg *Na*-APR-1 (M74). For all doses, the ratio of *Na*-APR-1 (M74) to Alhydrogel® will therefore remain constant: for the 30 and 100 µg doses of *Na*-APR-1 (M74) the respective amounts of Alhydrogel® will be 240 and 800 µg (corresponding to approximately 120 and 400 µg aluminum, respectively). *Na*-APR-1 (M74) protein expression was completed at Fraunhofer Center for Molecular Biotechnology (FhCMB/Newark, DE) and purification and vialing at the Walter Reed Army Institute of Research (WRAIR/Silver Spring, MD). The product conforms to established requirements of purity, sterility, safety, and identity. In this study, each dose of *Na*-APR-1 (M74)/Alhydrogel® will be administered in combination with 5 µg GLA-AF.

5.1.2.2 *Na*-GST-1/Alhydrogel®

Na-GST-1/Alhydrogel® is supplied as a sterile milky-white suspension (when shaken slightly). Each 2.0 ml vial contains 1.35 ml of a 0.1 mg/ml suspension of *Na*-GST-1 adsorbed to 0.8 mg/ml of Alhydrogel® in a buffer consisting of 10% glucose and 10 mM imidazole, pH 7.4. Glucose acts as an excipient and imidazole as the buffer based on evidence that these components specifically

enhance the stability and solubility of *Na*-GST-1. The maximum dose that will be administered is 100 µg of *Na*-GST-1, or 1.0 ml of the final drug product. This volume contains the equivalent of approximately 400 µg aluminum. Lower doses of *Na*-GST-1 are delivered by injecting smaller volumes of the 0.1 mg/ml suspension: for example, 0.3 ml will be injected to deliver 30 µg *Na*-GST-1. For all doses, the ratio of *Na*-GST-1 to Alhydrogel® will therefore remain constant: for the 30 and 100 µg doses of *Na*-GST-1 the respective amounts of Alhydrogel® will be 240 and 800 µg (corresponding to approximately 120 and 400 µg aluminum, respectively). *Na*-GST-1/Alhydrogel® was manufactured, formulated and vialed at Aeras Global Vaccine Foundation (Rockville, Maryland, USA).

5.1.2.3 Glucopyranosyl-Lipid A Aqueous Formulation (GLA-AF)

GLA-AF will be supplied to the trial site as a 0.5 mL aqueous solution in multi-dose vials containing 25 µg/mL of GLA without preservative. Appropriate volumes of GLA-AF will be withdrawn from the multi-dose vials using a syringe and added to a vial containing *Na*-APR-1 (M74)/Alhydrogel®, or vice versa (described in the Investigator's Brochure). The mixture must be administered not more than 24 hours after mixing the GLA-AF with *Na*-APR-1 (M74)/Alhydrogel®.

5.1.2.4 Saline Placebo

Sterile isotonic (0.9%) sodium chloride solution ("normal saline") for injection will be procured by the study site. Normal saline is a clear liquid. In study subjects randomized to receive Hepatitis B vaccine, normal saline will be administered intramuscularly as placebo in an equal volume to the Hepatitis B vaccine. It will be administered in the deltoid muscle of the arm opposite to the one in which the Hepatitis B vaccine is delivered so that all study subjects receive two injections on each day of vaccination, thus maintaining the study blind.

5.1.2.5 Hepatitis B Vaccine

The Hepatitis B vaccine is a non-infectious subunit viral vaccine derived from hepatitis B surface antigen (HBsAg) produced in yeast cells. The antigen is harvested and purified from fermentation cultures of a genetically modified strain of yeast containing the gene for HBsAg. The purified protein is then adjuvanted with aluminum hydroxide adjuvant. The hepatitis B vaccine is a slightly turbid sterile suspension for intramuscular injection. The vaccine is supplied in single-dose vials, with each vial containing enough vaccine to deliver one 0.5 mL dose. Each 0.5 mL dose contains 10 µg of HBsAg and 250 µg of aluminum as aluminum hydroxide. The product conforms to established requirements for sterility, safety, and identity.

5.1.3 Product Storage and Stability

Vaccines, GLA-AF, and placebo will be stored at the site in a refrigerator at 2°C to 8°C until just prior to administration and will not be frozen; refrigerator temperature will be monitored continuously. All vaccine vials will be stored in the upright position.

5.2 Dosage, Preparation and Administration of Study Intervention/Investigational Product

Doses of *Na*-GST-1/Alhydrogel® will be prepared by withdrawing appropriate volumes into syringes of appropriate size. The *Na*-APR-1 (M74)/Alhydrogel® plus GLA-AF formulations will be prepared by adding appropriate volumes of GLA-AF solution to vials of *Na*-APR-1 (M74)/Alhydrogel®, or vice versa, within 24 hours of vaccination. Doses of the Hepatitis B vaccine and sterile saline control will be prepared by withdrawing 0.5 mL into syringes of appropriate size.

Vaccine doses will be administered by qualified study personnel (a study nurse who will not be involved in further assessment of subjects) in the deltoid muscle of the appropriate arm after disinfecting the skin with an alcohol swab and allowing it to dry.

5.3 Modification of Study Intervention/Investigational Product for a Participant

There will be no dose modifications. If a child's second and/or third dose of study vaccination is deferred, it should be rescheduled to occur within the acceptable protocol-specified window for that visit. If this period elapses, the site must obtain prior approval from the Sponsor to administer the second and/or third study vaccination and the documentation of approval should be filed in the child's chart. Children who do not receive the second and/or third study vaccination will be asked to return for safety assessments and for scheduled venous blood sample collections for immunogenicity testing and will be followed for the duration of the study.

Unblinding can occur upon request of the Independent Medical Monitor or the SMC at any time during the study.

5.4 Accountability Procedures for the Study Intervention/Investigational Product(s)

Study vaccines, GLA-AF, and sterile saline placebo supplies must be received by a designated person at the study site, handled and stored safely and properly, and kept in a secure location to which only the designated individuals have access. Study-site personnel are responsible for maintaining accurate records of the vaccine supplies (i.e., *Na*-GST-1/Alhydrogel®, *Na*-APR-1 (M74)/Alhydrogel®, Hepatitis B vaccine, the sterile saline placebo, and GLA-AF) received, the quantities administered to study subjects, and the amounts remaining at the conclusion of the study.

After administration of vaccine or GLA-AF doses, the empty or wasted vials will be accounted for and stored at the study site until monitoring by the study Sponsor or their designee. At the conclusion of the study, all used and unused *Na*-GST-1/Alhydrogel®, *Na*-APR-1 (M74) /Alhydrogel®, and GLA-AF vials will be returned to the Sponsor or destroyed on site upon direction from the Sponsor, or maintained at 2 to 8ºC until further notice from the Sponsor regarding their disposition.

5.5 Concomitant Medications/Treatments

Administration of any medications, therapies, or vaccines will be recorded on the appropriate data collection form. Concomitant medications will include all medications taken within the 30 days prior to enrollment, 28 days after each dose of study product, and for new-onset chronic medical

conditions through the end of participation in the study for each subject. Prescription and over-the-counter drugs will be included as well as vitamins and supplements.

Use of new medication will prompt an evaluation for the presence of a new diagnosis of chronic medical disease or chronic medical condition.

Medications that might interfere with the evaluation of the investigational product should not be used unless absolutely necessary. Medications in this category include, but are not limited to, glucocorticoids, i.e., high dose oral, parenteral and daily inhaled steroids, and immunosuppressive or cytotoxic drugs. Other than from participation in this study, subjects should not receive experimental agents, including vaccines, for the duration of the study.

The administration of licensed vaccines, should be delayed until 28 days after the last administration of the investigational vaccine.

6 STUDY SCHEDULE

The following sections provide a detailed listing of the procedures and tests to be performed in this study at designated time points. The total volume of blood (approximately 94 mL) to be collected from each participating child over the duration of the trial (which is 13-15 months, depending on the vaccination schedule) is approximately the volume collected when donating one unit of blood and should not compromise the health of the children. See **Appendix A** for the schedule of study procedures.

6.1 Screening

The following procedures will be performed upon initial screening (note that all procedures might not be performed on the same day):

1. Explain the study, Informed Consent and the child assent form to the child's parent/legal guardian.
2. Ensure the child's parent/legal guardian has passed the informed consent comprehension questionnaire, has signed the Informed Consent as well as the child assent form and receives a signed copy of the Informed Consent.
3. Elicit a complete medical history, including medication history, for the child.
4. Administer a physical examination, including vital signs (heart rate, respiratory rate, arterial blood pressure, and a tympanic temperature).
5. Obtain blood for hematology, biochemistry, and serologic tests for HIV and viral hepatitis (B and C).
6. Obtain fecal sample for examination for ova and parasites.
7. Obtain urine for urine dipstick testing and examination for ova.
8. Obtain urine for metabolomics study.

Screening steps 3-7 must be performed within 90 days before the planned enrollment into the study. Should this screening window be exceeded before the first vaccination, screening procedures (not including administration of the informed consent form or comprehension questionnaire) may be repeated to ensure continued eligibility for the study (screening procedures can be repeated a maximum of one time). Screening laboratory tests (other than serologic tests for HIV and viral hepatitis) may be repeated up to one time to confirm any abnormalities.

If the fecal exam performed during screening demonstrates infection with an intestinal parasite the child will be treated with an appropriate medication prior to being enrolled in the study and before any vaccinations. Children infected with *Ascaris lumbricoides,* hookworm or *Trichuris trichiura* will be treated with three daily 400 mg doses of albendazole; children infected with *S. intercalatum* or *Taenia* spp. will be treated with a single 40 mg/kg oral dose of praziquantel; and individuals infected with *Strongyloides stercoralis* will be treated with three 400 mg daily oral doses of albendazole. If the urine exam performed during screening demonstrates infection with *Schistosoma haematobium*, infected children will be treated with a single 40 mg/kg oral dose of praziquantel. Children who have been treated for any parasitic infections, cannot be enrolled for vaccination within 14 days after the last day of treatment.

6.2 Enrollment/Baseline

Children will not be considered enrolled in the study until they have received their first dose of vaccine. In the event that a volunteer is randomized but not enrolled on the day of first vaccination, they will be replaced with an eligible alternate.

Study Day 0 (Day of First Vaccination)
1. Verify that Informed Consent was obtained from the child's parent/guardian.
2. Verify that all applicable eligibility criteria have been met.
3. Perform abbreviated history (including concomitant medications) and physical exam, focusing on any acute complaints.
4. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
5. Obtain a urine sample for metabolomics study.
6. Record vital signs (blood pressure, a tympanic temperature, and heart rate).
7. Administer the vaccines.
8. Observe for at least 1 hour after vaccination to evaluate for immediate adverse reactions. During the 1-hour post-immunization wait period, study staff will discuss signs and symptoms of potential AEs with parent/guardian and child.

6.3 Follow-up

Study Day 1
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints (i.e., AE data collection).
2. Record vital signs.
3. Obtain a urine sample for metabolomics study.

Study Day 3 +/- 1
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain a urine sample for metabolomics study.

Study Day 7 +/- 1
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) antibody assays and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 14 +/- 2
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.

3. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 28 +/- 4
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 56 +/- 7 (Day of Second Vaccination)
1. Perform basic history (including concomitant medications) and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain a urine sample for metabolomics study.
4. Record vital signs.
5. Administer the vaccine.
6. Observe for at least 1 hour after vaccination to evaluate for immediate adverse reactions.

Study Day 57 (1 day after Second Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.

Study Day 59 (3 +/- 1 days after Second Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain a urine sample for metabolomics study.

Study Day 63 (7 +/- 1 days after Second Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 70 (14 +/- 2 days after Second Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 84 (28 +/- 4 days after Second Vaccination)
1. Perform basic history and physical exam, emphasizing examination of any complaints.
2. Record vital signs.
3. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 112 +/- 14 [Groups 1a, 2a, & 3a] or 180 [Groups 1b, 2b, & 3b]
(Day of Third Vaccination)
1. Perform basic history (including concomitant medications) and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain a urine sample for metabolomics study.
4. Record vital signs.
5. Administer the vaccine.
6. Observe for at least 1 hour after vaccination to evaluate for immediate adverse reactions.

Study Day 113/181 (1 day after Third Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain a urine sample for metabolomics study.

Study Day 115/183 (3 +/- 1 days after Third Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain a urine sample for metabolomics study.

Study Day 119/187 (7 +/- 1 days after Third Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 126/194 (14 +/- 2 days after Third Vaccination)
1. Perform basic history and physical exam (including injection site), emphasizing examination of any acute complaints.
2. Record vital signs.
3. Obtain blood for hematology, biochemistry, anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
4. Obtain a urine sample for metabolomics study.

Study Day 140 (28 +/-4 days after Third Vaccination) [Groups 1a, 2a, & 3a ONLY]
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) antibody assays and cellular immunology assays.
3. Obtain stool for examination for ova and larvae from parasites.
4. Obtain a urine sample for metabolomics study.

Study Day 200 (3 months +/-14 days after Third Vaccination) [Groups 1a, 2a, & 3a ONLY]
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain a urine sample for metabolomics study.

Study Day 208 (28 +/-4 days after Third Vaccination) [Groups 1b, 2b, & 3b ONLY]
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain stool for examination for ova and larvae from parasites.
4. Obtain a urine sample for metabolomics study.

Study Day 268 (3 months +/-14 days after Third Vaccination) [Groups 1b, 2b, & 3b ONLY]
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain a urine sample for metabolomics study.

Study Day 290/358 (6 months +/-14 days after Third Vaccination)
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain blood for anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) humoral and cellular immunology assays.
3. Obtain a urine sample for metabolomics study.

6.4 Final Study Visit

Study Day 380/448 (9 months +/-21 days after Third Vaccination)
1. Perform basic history and physical exam, emphasizing examination of any acute complaints.
2. Obtain stool for examination for ova and larvae from parasites.

6.5 Early Termination Visit

The following activities will be performed at the early termination visit for study participants who withdraw, or are withdrawn or terminated from the study:

- Obtain interim medical history by interview of subjects and note any changes since the previous visit.

- All concomitant medications will be recorded on the appropriate data collection form (if within 28 days of child's last vaccination received in the study).

- Information regarding adverse events/Serious Adverse Events will be assessed and recorded on the appropriate data collection form (adverse events will be limited to new-onset chronic medical conditions and SAEs if after 28 days after the third study vaccination).

- A targeted physical examination may be, if indicated based on review of interim medical history.

- Approximately 2.4 mL of venous blood may be collected for safety labs and performed by the clinical laboratory (if prior to the post-vaccination day 14 visit and if not recently obtained per study schedule).

- Approximately 6.2 mL of venous blood may be collected for antibody and cellular assays (if prior to post-vaccination day 14 visit and if not recently obtained).

6.6 Unscheduled Visit

Unscheduled visits may occur at any time during the study. Any of the following activities may be performed:

- Review concomitant medications (if started prior to 28 days after a study vaccination).

- Review adverse events (if started prior to 28 days after a study vaccination).

- Review serious adverse events and new-onset chronic medical conditions

- Obtain medical history by interview of children and their parent/guardian and note any changes since the previous visit (if indicated).

- A targeted physical examination may be performed, if indicated based on review of medical history.

- Examine study vaccination site (if within 14 days after a study vaccination).

- Approximately 2.4 mL of venous blood will be collected for safety labs and performed by the study clinical laboratory (if indicated).

7 STUDY PROCEDURES/EVALUATIONS

7.1 Clinical Evaluations

Children will be monitored for local and systemic adverse events during specific protocol-defined post-vaccination periods. As with other aluminum hydroxide-adsorbed vaccines, hypersensitivity reactions would be expected to occur within the first 24 hours after receipt of either of the two vaccines, and other severe local or systemic reactions within 72 hours of vaccination. Children will therefore be observed for immediate reactions following each vaccination for at least 1 hour, and will have a clinical assessment either in their home or at the study clinic on Days 1, 3, 7, 14 and 28 following each vaccination. If required, transportation to and from the clinic will be provided. Study clinicians will also stay in or near the study site for the duration of the trial and will be available to study subjects at all times. Should the parent/guardian of a child participating in the study call on a study clinician to report an adverse event (AE), it will be fully documented in their study chart, and discussed with the Principal Investigator.

All AEs will be graded for severity and relationship to study vaccine, captured on the appropriate case report form (CRF), and followed to resolution. All Serious Adverse Events (SAEs) will be reviewed by a study physician, recorded on the appropriate SAE form, reported according to applicable regulations and guidelines, and followed through to resolution or stabilization by a study physician. Special attention will also be paid to monitoring for the occurrence of Adverse Events of Special Interest (AESIs), which include inflammatory and autoimmune disorders that may potentially be related to the use of an immunostimulatory adjuvant (although none have been associated with the use of GLA-AF, to date).

A local Independent Medical Monitor will be appointed and a Safety Monitoring Committee (SMC) formed to monitor subject safety and to advise the Principal Investigator and co-investigators on trial-related medical questions or problems. The SMC will periodically review data on safety and enrollment, and will review cumulative safety data for evidence of study related AEs, adherence to the protocol, and factors that may affect outcome or study data such as protocol violations and losses to follow up.

7.2 Laboratory Evaluations

7.2.1 Clinical Laboratory Evaluations

Using standard techniques, the following tests will be performed at the CERMEL clinical laboratory in Lambaréné, Gabon. One or more of the laboratory parameters may be repeated at any time during the study as determined by the PI, if indicated by an AE. A clinically significant abnormal value should be repeated within 14 days if possible and followed up as clinically relevant.

1. Complete blood count plus white blood cell differential (WBC, absolute neutrophil count [ANC], hemoglobin concentration and platelet count)
2. Serum creatinine
3. Alanine aminotransferase (ALT)
4. HBsAg rapid test
5. HCV rapid test
6. HIV rapid test

Urine dipstick testing will also be performed at the trial sites using an approved product.

7.2.2 Special Assays or Procedures

Anti-*Na*-GST-1 and anti-*Na*-APR-1 antibody assays (e.g., ELISAs and functional antibody assays) will be performed at the Department of Microbiology, Immunology and Tropical Medicine of the George Washington University (Washington, DC, USA). Cellular immunological responses and metabolomics studies will be performed at CERMEL and at the University of Leiden (Leiden, The Netherlands). Cryopreservation of sera, plasma and PBMCs will be at -80°C or in liquid nitrogen.

7.2.2.1 Anti-*Na*-APR-1 (M74) and Anti-*Na*-GST-1 Antibody Assays

Antibodies to *Na*-GST-1 and *Na*-APR-1 will be measured in serum or plasma of study subjects using an indirect ELISA assay. Antigen-specific IgG and IgE antibody levels will be measured at baseline and at several time-points post-vaccination. In addition, antigen-specific IgG subclasses (IgG1-4) will be measured by indirect ELISA. For the ELISA method, microwell plates will be coated with purified recombinant *Na*-GST-1 or *Na*-APR-1 (M74), blocked, and incubated with serial dilutions of a positive “standard reference serum” pool to generate a reference curve from which antibody levels of test sera can be interpolated. After washing, a horseradish peroxidase conjugated anti-human antibody will be added and incubated with on the plates, washed again, incubated with a chromogenic substrate, and antibody levels measured on an ELISA plate reader.

7.2.2.2 Enzyme Neutralization Assays

In vitro assays have been developed to measure the functional capacity of antibodies induced by vaccination with recombinant *Na*-GST-1 or *Na*-APR-1 (M74) to inhibit or neutralize the activity of the native forms of these proteins. Serum or plasma samples collected from study subjects will be tested for their neutralizing capacity at several post-vaccination time points. These assays will be performed at the Clinical Immunology Laboratory of the Department of MITM of the George Washington University.

7.2.2.3 Cellular Immunology Assays

Cytokine and chemokine assays will be performed on supernatants from cryopreserved PBMCs collected at various time points in the study, by separating PBMCs from whole blood and culturing cells with media only, the mitogen phytohemagglutinin, the antigens *Na*-APR-1 (M74) or *Na*-GST-

1, and crude *N. americanus* antigens extracts (e.g., adult somatic crude antigen extract). Cytokines (e.g., IL-2, IL-4, IL-5, IL-10, TNFα, and IFNγ) will be quantified using Cytometric Bead Analysis kits of supernatants collected from PBMCs stimulated with *Na*-APR-1 (M74) or *Na*-GST-1 antigen for 48 and 72 hours *in vitro*. Chemokines (e.g., RANTES, MIP1a, MCP-1, and IP-10) will be quantified in supernatants using commercially available ELISA kits.

7.2.2.4 Parasitology Assays

Fecal samples will be tested for helminth ova or larvae by the Kato Katz fecal thick smear technique.

7.2.2.5 Metabolomic Assays

Time-course of metabolic change after vaccination using urine will be performed by 1H-NMR and LC-MS metabolomics approaches that use the NMR and mass spectrometry platforms in LUMC. Non-invasive urine sampling will be undertaken at regular time points when study participants are seen before and after vaccination.

In order to assess the metabolite changes after the administration of anthelmintic drugs, urine sampling will be undertaken at screening, for baseline measurements. For children who need albendazole or praziquantel treatment for helminthic infections, and are included for vaccination, the urine sampling will be repeated the day of vaccination, to measure the metabolite changes. Samples will be coded with the child's screening number. For children who will not be enrolled for vaccination, obtained urine samples will be destroyed.

The metabolomics studies are included as exploratory endpoints. Metabolomics involves profiling and measuring small molecules that reflect the metabolic response at the cellular, tissue and organ level, and can provide insight into the functioning and induction of the immune response. Two metabolomic studies in infection models have revealed novel insights into how lipid related metabolites play an important role in viral (Wikoff, 2009) and bacterial (Tobin, 2012) infections. Importantly, work has shown that the metabolic state of immune cells can have far reaching consequences for the development of long-term memory responses (Pearce, 2009). Therefore, whole metabolome measurements will be made in urine and both untargeted and pathway specific analysis will be applied to identify biomarkers and also provide insight into mechanisms of successful vaccination as determined by the neutralizing capacity of antibodies raised to Na-APR-1 and Na-GST-1. These ancillary cellular and metabolic studies in this Phase 1 trial will generate data that will be correlated with i) the development of neutralizing antibodies to Na-GST-1 and Na-APR-1, in order to understand which processes are important for immunogenicity of the vaccine and successful generation of neutralizing antibodies; and, ii) the clinical data obtained in order to delineate mechanisms underlying any potential adverse reactions.

7.2.3 Specimen Preparation, Handling, and Shipping

7.2.3.1 Instructions for Specimen Preparation, Handling, and Storage

Instructions for specimen preparation, handling, and storage are included in the protocol-specific Manual of Procedures, as appropriate.

7.2.3.2 Specimen Shipment

Specimen shipment will occur at intervals during the course of the study following all applicable International Air Transport Association (IATA) requirements and according to the specifics for storage temperature and documentation as detailed in protocol-specific SOPs, as appropriate.

Specimens for immune responses will be shipped from the study site at CERMEL to the George Washington University in Washington, DC, and to the LUMC in Leiden, The Netherlands, in a blinded manner in batches as they become available.

8 ASSESSMENT OF SAFETY

8.1 Specification of Safety Parameters

Safety will be assessed by the frequency and severity of:

1. Study vaccine-related serious adverse events occurring from the time of the first study vaccination through approximately 9 months after the last study vaccination.

2. Solicited Adverse Events – reactogenicity events occurring on the day of each study vaccination through 14 days after each study vaccination:
   a) Injection site reactions including erythema (redness), induration (hardness)/swelling, pain, and tenderness.
   b) Systemic reactions including fever, myalgia (body aches/muscular pain exclusive of the injection site), arthralgia (joint pain exclusive of the injection site), headache, urticaria, vomiting, and nausea.

3. Clinical safety laboratory adverse events occurring from the time of each study vaccination through approximately 14 days after each set of vaccinations. Parameters to be evaluated include WBC, ANC, hemoglobin concentration, and platelet count; ALT; and, creatinine.

4. Unsolicited Adverse Events – non-serious adverse events occurring from the time of teach study vaccination through approximately 28 days after each vaccination.

5. New-onset chronic medical conditions occurring from the time of the first study vaccination through approximately 9 months after the last study vaccination.

6. Adverse Events of Special Interest occurring from the time of the first study vaccination through approximately 9 months after the last study vaccination.

8.2 Methods and Timing for Assessing, Recording, and Analyzing Safety Parameters

8.2.1 Adverse Events

An adverse event (AE) includes any noxious, pathological or unintended change in anatomical, physiological or metabolic functions as indicated by physical signs, symptoms and/or laboratory-detected changes occurring in any phase of the clinical study, whether associated with the study vaccine or placebo, and whether or not considered vaccination related. This includes an exacerbation of pre-existing conditions and intercurrent illnesses.

All AEs will be graded for severity and relationship to study vaccine. A study clinician will be readily available for the duration of the trial to assess AEs. Should a parent/guardian or child call on a study clinician to report an AE, it will be fully documented in their study chart, and discussed with the Principal Investigator.

All AEs will be assessed by the investigator using the following protocol-defined grading system, as or described in Tables 2-5:

Grade 1:	**Mild** - No effect on activities of daily living; no medical intervention/therapy required
Grade 2:	**Moderate** - Partial limitation in activities of daily living (can complete ≥50% of baseline); no or minimal medical intervention/therapy required
Grade 3:	**Severe** - Activities of daily living limited to < 50% of baseline; medical evaluation/therapy required

All AEs will have their possible relationship to study vaccine assessed using the following terms:

Definite:	Clear-cut temporal association, and no other possible cause.
Probable:	Clear-cut temporal association and a potential alternative etiology is not apparent.
Possible:	Less clear temporal association; other etiologies also possible.
Unlikely:	Temporal association between the AE and the vaccine or the nature of the event is such that the vaccine is not likely to have had any reasonable association with the observed illness/event (cause and effect relationship improbable but not impossible).
Not Related:	The AE is completely independent of vaccine administration; and/or evidence exists that the event is definitely related to another etiology.

The degree of certainty with which an AE can be attributed to administration of study vaccine will be determined by how well the event can be understood in terms of one or more of the following:

1. The event being temporally related with vaccination or reproduced on re-vaccination.
2. A reaction of similar nature having previously been observed with this type of vaccine and/or formulation.
3. The event having often been reported in the literature for similar types of vaccines.

8.2.2 Reactogenicity

Reactogenicity events are AEs that are known to occur with types of vaccine similar in composition to those being tested in this study. The following Toxicity Grading Scales will be used to grade solicited local (injection site) and systemic (subjective and quantitative) reactions:

Table 2: Assessment of Solicited or Expected Adverse Event Severity

Adverse Event	Grade	Severity
Pain/tenderness at injection site	1 2 3	Easily tolerated, does not interfere with activity Limits use of limb or interferes with daily activity Prevents daily activity
Erythema at injection site	1 2 3	< 10 mm 10 mm – 25 mm > 25 mm
Induration/swelling at injection Site	1 2 3	< 10 mm 10 mm – 25 mm > 25 mm
Fever (tympanic)	1 2 3	38.0°C – 38.4°C 38.5°C – 38.9°C >39.0°C
Headache	1 2 3	Easily tolerated, does not interfere with daily activity Responds to non-narcotic pain reliever or interferes with daily activity Responds to narcotic pain reliever or prevents daily activity
Nausea	1 2 3	Easily tolerated, does not interfere with daily activity Decreased oral intake Minimal oral intake
Vomiting	1 2 3	1-2 episodes in 24 hours > 2 episodes in 24 hours Prevents daily activity or requires outpatient IV hydration
Myalgia	1 2 3	Easily tolerated, does not interfere with daily activity Interferes with daily activity Prevents daily activity
Arthralgia	1 2 3	Easily tolerated, does not interfere with activity Interferes with daily activity Prevents daily activity
Urticaria	1 2 3	Requiring no medications Requiring oral or topical treatment, or IV medication or steroids for <24 hours Requiring IV medication or steroids for >24 hours

Table 3: Assessment of Unsolicited Systemic Adverse Event Severity

Systemic AE	Mild (Grade 1)	Moderate (Grade 2)	Severe (Grade 3)
Anorexia	Decreased appetite without decreased oral intake lasting greater than 48 hours	Decreased appetite associated with decreased oral intake without significant weight loss	Loss of appetite associated with significant weight loss
Diarrhea	3 loose stools/24 hours	Liquid stools	Liquid stools > 4x the amount or number normal for this child
Constipation	Not Applicable	Hard, dry stools with a change in frequency	Resulting in abdominal pain
Fatigue	No interference w/activity	Some interference w/activity	Significant, prevents daily activity

Arthritis	Mild pain with inflammation, erythema or joint swelling – but not interfering with function	Moderate pain with inflammation, erythema or joint swelling – interfering with function, but not with activities of daily living	Severe pain with inflammation, erythema or joint swelling –and interfering with activities of daily living
Vasovagal episode (associated with a procedure of any kind)	Present without loss of consciousness	Present with transient loss of consciousness	Not Applicable
Cough	Transient- no treatment	Persistent cough; treatment responsive	Paroxysmal cough; uncontrolled with treatment
Bronchospasm, Acute	Transient; no treatment; 70% - 80% FEV_1 of peak flow	Requires treatment; normalizes with bronchodilator; FEV_1 50% - 70% (of peak flow)	No normalization with bronchodilator; FEV_1 25% - 50% of peak flow; or retractions present
Dyspnea	Dyspnea on exertion	Dyspnea with normal activity	Dyspnea at rest
Hypersensitivity	Transient flushing or pruritus without rash	Pruritic rash or mild urticaria	Symptomatic bronchospasm, with or without urticarial

Table 4: Assessment of Basic Body Function Adverse Event Severity

Vital Signs*	**Mild (Grade 1)**	**Moderate (Grade 2)**	**Severe (Grade 3)**
Tachycardia – beats per minute	115-130	131-150	>150
Bradycardia – beats per minute	55-59	50-54	<50
Hypertension** (systolic, mm Hg)	126-135	136-145	>145
Hypertension** (diastolic, mm Hg)	86-90	91-96	>96
Hypotension	Symptomatic, oral fluids not required	Symptomatic, corrected with oral fluid replacement	Symptomatic, IV fluids indicated

**Subject should be at rest for measurement of vital signs*

***With repeat testing at same visit*

Table 5: Assessment of Laboratory Adverse Event Severity

HEMATOLOGY	**Grade 1 (Mild)**	**Grade 2 (Moderate)**	**Grade 3 (Severe)**
Hemoglobin	8.2 – 9.2 g/dL	7.1 – 8.1 g/dL	<7.0 g/dL
Platelets	110,000 – 130,000/mm^3	90,000 – 109,999/mm^3	<90,000/mm^3
WBCs *(increase)*	13,800 – 17,000/mm^3	17,001 – 20,000/mm^3	<20,000/mm^3
WBCs *(decrease)*	2500 – 4200/mm^3	1500 – 2499/mm^3	<1500/mm^3
ANC *(decrease)*	750 – 1000/mm^3	500 – 749/mm^3	<500/mm^3
CLINICAL CHEMISTRIES	**Grade 1**	**Grade 2**	**Grade 3**
Serum creatinine	1.2 – 1.4 mg/dL	1.5 – 1.7 mg/dL	>1.8 mg/dL or requires dialysis
ALT	48 – 105 U/L	106 – 220 U/L	>220 U/L

All local (injection-site) reactions occurring within the first 14 days after vaccination will be considered definitely related to vaccination.

8.2.3 Serious Adverse Events

An SAE is an AE, whether considered related to a study vaccine or not, meeting one of the following conditions:

1. Death during the period of protocol-defined surveillance
2. Life threatening: defined as an event that places a subject at immediate risk of death at the time of the event and does not refer to an event that hypothetically might have caused death were it more severe
3. Hospitalization during the period of protocol-defined surveillance: defined as at least an overnight stay in the hospital or emergency ward for treatment that would have been inappropriate if administered in the outpatient setting
4. Results in a congenital anomaly or birth defect
5. Results in a persistent or significant disability or incapacity: defined as a substantial disruption of the study subject's ability to carry out normal life functions
6. Any other important medical event that may not result in death, be life threatening, or require hospitalization, may be considered a serious AE when, based upon appropriate medical judgment, the event may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above.

8.2.4 Procedures to be Followed in the Event of Abnormal Laboratory Test Values or Abnormal Clinical Findings

The Principal Investigator or appropriate co-investigator is responsible for reporting all AE/SAEs that are observed or reported during the study, regardless of the relationship to study products. AE/SAEs, abnormal clinical laboratory test values, or abnormal clinical findings will be documented, reported, and followed appropriately.

8.3 Reporting Procedures

8.3.1 Serious Adverse Events

All SAEs will be reviewed by a study physician, recorded on the appropriate SAE form, and followed through to resolution or stabilization by a study physician. All SAEs will be reported to AIGHD within 1 working day of notification of the SAE occurrence to the Principal Investigator, via either:

- Notification of the SAE via the eDM[1] system (automatic updates are then provided to all relevant pharmacovigilance staff), OR
- Notification via a scanned PDF version of an SAE form to SAE@hookvac.eu. This distribution list will provide automatic distribution to all relevant pharmacovigilance staff.

In addition, the Principal Investigator will report all SAEs that are determined to be definitely or probably related to vaccination to the Institutional Review Board (IRB) that approved the study (i.e., the local IRB in Gabon) according to the IRB's reporting timelines and using the IRB-specified reporting forms, as well as to the Independent Medical Monitor.

All local and systemic reactions not meeting the criteria for SAE will be captured on the appropriate case report form (CRF). These events will be followed to resolution.

8.3.2 Regulatory Reporting

Following notification from the Principal Investigator, the IND sponsor (Sabin Vaccine Institute) will report events that are both serious and unexpected that are possibly, probably, or definitely related to the vaccine, to the FDA within the required timelines: fatal and life-threatening events within 7 calendar days (by phone, fax, or internet) and all other SAEs in writing within 15 calendar days. All SAEs not listed as possibly, probably, or definitely related will be reported to the FDA and ANVISA at least annually in a summary format.

All Adverse Events of Special Interest (AESIs; see below) will be reported to FDA according to the same procedure as for reporting SAEs, and according to the same timelines as described above.

[1] Electronic Data Management system

8.3.3 Adverse Events of Special Interest (AESI)

Special attention will be paid to monitoring for the occurrence of certain adverse events termed, "**Adverse Events of Special Interest**" or AESIs. These include inflammatory and autoimmune disorders that may potentially be related to the use of an immunostimulatory adjuvant (although none have been associated with the use of GLA-AF). The occurrence of the following AESI's will be closely monitored:

- Neuroinflammatory disorders (optic neuritis, multiple sclerosis, demyelinating disease, transverse myelitis, Guillain-Barré syndrome, myasthenia gravis, encephalitis, neuritis, Bell's palsy)
- Musculoskeletal disorders (systemic lupus erythematosus, cutaneous lupus, Sjogren's syndrome, scleroderma, dermatomyositis, polymyositis, rheumatoid arthritis, juvenile rheumatoid arthritis, polymyalgia, rheumatica, reactive arthritis, psoriatic arthropathy, ankylosing spondylitis, spondylarthropathy)
- Gastrointestinal disorders (Crohn's disease, ulcerative colitis, celiac disease)
- Metabolic diseases (autoimmune thyroiditis, Grave's or Basedow's disease, Hashimoto thyroiditis, insulin-dependent diabetes mellitus, Addison's disease)
- Skin disorders (psoriasis, vitiligo, Raynaud's phenomenon, erythema nodosum, autoimmune bullous skin diseases)
- Others (autoimmune hemolytic anemia, idiopathic thrombocytopenic purpura, antiphospholipid syndrome, temporal arteritis, Behcet's syndrome, pernicious anemia, autoimmune hepatitis, primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune glomerulonephritis, autoimmune uveitis, autoimmune cardiomyopathy, sarcoidosis, Stevens-Johnson syndrome).
- Vasculitides

8.3.4 Medically-Attended Adverse Events (MAAEs)

Special attention will also be paid to recording medically-attended adverse events, which are defined as any clinical symptom or diagnosis (including local symptoms at the injection site or systemic symptoms) for which medical evaluation and/or care is sought from a healthcare professional, outside of a regularly scheduled study visit. Note that MAAEs are not the same as SAEs. Whether or not an AE is an MAAE will be recorded on the appropriate Case Report Form.

8.4 Type and Duration of Follow-up of Subjects after Adverse Events

AEs will be recorded if they start from the time of the first study vaccination (Day 0) up to 28 days after each dose of study vaccine. AEs limited to new-onset chronic medical conditions will be followed through approximately 9 months after the third study vaccination (Day 380/448).

8.5 Halting Rules

If a dose of study vaccine is considered significantly reactogenic (see below and Section 8.6.2), dose escalation and/or additional vaccinations will be suspended until reviewed by the SMC, Independent Medical Monitor, and IND sponsor (SVI). Any recommendation of the Independent Safety Monitor and/or SMC to resume or suspend further injections (either for an individual subject, an entire Group, or the full study) will be communicated in writing to the sponsor and Principal Investigator. All communications from the SMC will subsequently be forwarded by the investigators to the IRB.

The following criteria will be used to define significant reactogenicity:

- One or more participants experience a protocol defined SAE that is determined to be possibly, probably, or definitely related to a study vaccine, **OR,**
- One or more participants experience a protocol defined AESI that is determined to be possibly, probably, or definitely related to a study vaccine, **OR,**
- One or more participants experience a protocol defined Grade 3 hypersensitivity reaction that is possibly, probably or definitely related to a study vaccine, **OR,**
- Two or more participants experience a protocol defined Grade 2 hypersensitivity reaction that is probably or definitely related to a study vaccine, **OR,**
- Three or more participants in a single Group (i.e., Group 1, Group 2, etc.) experience the same objective physical finding or laboratory abnormality of Grade 3 or higher (with the exception of isolated Grade 3 erythema or swelling), that is determined to be probably or definitely related to a study vaccine, **OR,**
- Three or more participants in a single Group experience the same Grade 2 or higher safety laboratory abnormality or Grade 3 clinical AE that is possibly, probably or definitely related to a study vaccine.

The study will be double-blinded until all study subjects have completed their Day 208 visit, the safety and secondary immunogenicity outcomes (i.e., anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) IgG antibody results by ELISA) have been monitored and entered into the database, and the database has been "soft-locked" for interim analysis. After this milestone, the Sponsor and investigators will be unblinded. During the double-blinded part of the study, a study subject's randomization code may be unblinded only for safety purposes. This is unlikely to occur, since once a vaccine is administered, knowing which vaccine was given is unlikely to influence the medical management of an AE. This procedure is therefore exceptional and any decision to unblind will be discussed with the sponsor, the Principal Investigator, the Independent Medical Monitor, and the SMC. If deemed necessary for urgent safety reasons, the Independent Medical Monitor, in consultation with the SMC (if possible in a timely manner), may unblind a specific subject without revealing the study blind to the investigators or the Sponsor. Any unblinding will be thoroughly documented. It is to be emphasized that the Independent Medical Monitor may put the study on hold at any time and discuss with the SMC. In the event that the investigators come to know the study code prior to final unblinding, the Principal Investigator must notify the Sponsor immediately. The reasons will be documented by the Principal Investigator and added to the study file.

The decision to completely unblind the study prior to Day 208 or permanently stop the study prior to the final study visit of all participating children will take the form of a formal recommendation by the SMC to the study Sponsor. The Principal Investigator must then notify the IRB of this decision.

8.6 Safety Oversight

8.6.1 Independent Medical Monitor (IMM)

An independent Medical Monitor will be appointed for oversight of subject safety in this trial. The IMM will be a local, qualified physician who will be available to advise the investigators on trial-related medical questions or problems. The IMM will work with the study team to ensure adequacy of adverse event monitoring and reporting. Should the IMM not be available, he/she will recommend an alternative to serve as a substitute IMM.

The IMM's primary responsibility will be to monitor subject safety. The Principal Investigator is responsible for ensuring that the IMM is aware of any new safety information that becomes available during the course of the trial.

8.6.2 Safety Monitoring Committee (SMC)

At least three individuals will be selected to serve as the study Safety Monitoring Committee (SMC) to advise the Sponsor and the study investigators on the trial. All SMC members will be independent from the Sponsor and study site. The SMC's primary responsibility will be to monitor subject safety. The Principal Investigator is responsible for ensuring that the SMC is aware of all new safety information. The SMC will periodically review data on safety and enrollment, and will review cumulative safety data for evidence of study related AEs, adherence to the protocol, and factors that may affect outcome or study data such as protocol violations and losses to follow up.

Cumulative safety data reports from the trial will be submitted to the SMC before beginning vaccinations in Group 2 and Group 3 of the study. These safety data reports will include data from at least the first 14 days after first vaccination of all subjects in the prior Group. After the third and final vaccination has been administered to all cohorts, additional safety and immunology results and reports will be submitted to the SMC as they become available.

Conference calls between the investigators and the SMC will be scheduled at least one week prior to beginning vaccinations in Group 2 and Group 3 of the study. If no criteria for halting the study are met (see Section 8.5 above) vaccinations will proceed with approval from the SMC.

Written approval (via fax or email) to proceed to vaccinating Group 2 and Group 3 must be obtained from the SMC prior to vaccine administration. Both the SMC and IMM will have access to the randomization code, as they may wish to review the data in an unblinded fashion should significant safety questions arise prior to the final unblinding of the study.

It is the Principal Investigator's (or designated agent) responsibility to ensure that the SMC reviews the current safety data (grouped by dose cohort), study protocol, and any other requested documents at its meetings. Occurrence of an SAE will be reported to the SMC at the same time

that it is reported to the IRB. Additionally, any new information that may adversely affect the safety of the subjects or the conduct of the study will be submitted to the SMC as it becomes available.

9 CLINICAL MONITORING

9.1 Site Monitoring Plan

The Sponsor (or its designee) will monitor all aspects of the study, with respect to current Good Clinical Practices, and for compliance with applicable government regulations. Prior to the start of the study, the Principal Investigator will be informed of the frequency of monitoring visits and will be given reasonable notification prior to each visit. The objectives of a monitoring visit will be to verify the prompt reporting of SAEs, to check the availability of the signed Informed Consent and the child assent form for enrolled study subjects, to compare CRFs and spreadsheets with source data for completeness and accuracy, to verify compliance with the clinical protocol, and to check investigational product accountability. During the monitoring visit, the Principal Investigator (and/or designee) and other study personnel should be available to discuss the study. Study documents must be available for review throughout the course of the study.

10 STATISTICAL CONSIDERATIONS

10.1 Sample Size Considerations

This Phase 1 trial is not powered to detect statistically significant differences between groups. Even though comparative statistics for the safety variables will be computed, the study will have low power to detect anything other than very large differences in the incidence of local injection site and systemic side effects between the vaccination groups. This is done weighing the need to detect any possible untoward reactions against the need to limit the number of volunteers involved for safety purposes. The sample size of 60 for this study is within the range commonly used in Phase 1 trials for the initial assessment of the safety, tolerance and immunogenicity of investigational vaccines.

The primary objective of the study is to estimate the frequency of vaccine-related adverse events. Therefore, rather than providing a statistical power calculation for a specific hypothesis, we illustrate below the probability of observing one or more events, such as an adverse event of a particular type, for a single vaccine dose (N=20), formulation (N=30), or for all participants (N=60) under different assumptions of the true rate at which such events occur in the population.

	Probability of Observing 1 or More Events		
	N=20	**N=30**	**N=60**
"True" (but unknown) Probability of an Event			
0.5%	9.54%	13.96%	25.97%
1.0%	18.21%	26.03%	45.28%
3.0%	45.62%	59.90%	83.92%
5.0%	64.15%	78.54%	95.39%
10.0%	87.84%	95.76%	99.82%

As illustrated in the table above, the study will have high probability (>= 80%) of detecting events that are commonly occurring in the population. For example, with a sample of 20 participants (which is equivalent to the size of each dose group of *Na*-APR-1 (M74) in this study), there is an 87.84% probability of observing 1 or more events when the events occur at a rate of 10.0% in the population. As the sample size increases, the study is able to detect events that occur at lower rates. For example, using a sample size of 30 participants (which is equivalent to the number of participants assigned to vaccine co-administration in this study vs. *Na*-APR-1 (M74) alone), there is a 78.54% probability of observing 1 or more events that occur at a rate of 5% in the population. Using a sample of size 60 (equivalent to the entire study population) there is an 83.92% probability of observing 1 or more events that occur at a rate of 3% in the population. Detection of rare events—those that occur at a rate of 1% or less in the population--is not likely in this study.

10.2 Planned Interim Analyses

See section 14.4 for timing of reports.

10.2.1 Safety Review

Descriptive and hypothesis-testing approaches will be used to estimate AE rates and to compare these rates in the difference doses and formulations of *Na*-APR-1 (M74) alone or co-administered with *Na*-GST-1. Estimates will be presented with their 95% confidence intervals. Formal statistical tests, as outlined below, will be used to compare doses. No formal adjustments for multiple comparisons will be made. Statistical tests will use a two-sided significance level of 5%.

The proportion of subjects with at least one injection site AE will be compared by vaccine allocation (e.g., *Na*-APR-1 (M74) co-administered with *Na*-GST-1 vs. hepatitis B vaccine), by and by *Na*-APR-1 (M74)/*Na*-GST-1 dose.

Laboratory results (hematological and clinical chemistry) will be examined for trends over time and any clinically significant values for individuals will be reported.

10.2.2 Immunogenicity Review

The proportion of subjects with detectable anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) antibody responses, and the duration of these responses, will be summarized as descriptive measures. Geometric mean antibody responses will be compared between dose groups for each vaccine antigen with comparisons made by analysis of variance (ANOVA) tests and pair-wise comparisons by contrasts. Antibody responses will be measured at Days 0, 7, 14, 28, 56, 63, 70, 84, 112/180, 119/187, and 126/194 for interim analysis, with Days 140/208, 200/268, 290/358, and 380/448 added for final analysis.

To exploit the multiple measures of antibody within each subject, a longitudinal model may be built (if possible) to describe the antibody responses over time. The models will explore if there are any differences between those administered *Na*-APR-1 (M74)/Alhydrogel® co-administered *Na*-GST-1/Alhydrogel® and those administered the hepatitis B vaccine.

Summary statistics will be used to present exploratory study results of the cellular immune responses to *Na*-GST-1 and *Na*-APR-1 (M74) before and after immunization. To assess the changes in lymphocyte proliferative responses and cytokine/chemokine profiles over time, a generalized estimating equations model with robust standard errors will be constructed. When necessary, data reduction approaches will be used.

10.3 Final Analysis Plan

The purpose of this trial is to estimate AE rates and patterns of immune response as well as to compare these rates and patterns when *Na*-GST-1/Alhydrogel® and *Na*-APR-1 (M74)/Alhydrogel® are administered separately or when they are co-administered.

This section briefly describes the statistical methods to be used; a detailed analytical plan will fully describe the methods. The analytical plan will discuss the planned approaches to missing data. Deviations from the original analytical plan will be thoroughly documented and reported to the Sponsor.

Descriptive and hypothesis-testing approaches will be used to meet the protocol objectives as stated in **Section 3**. Estimates will be presented with their 95% confidence intervals. Formal statistical tests, as outlined below, will be used to compare doses. Statistical tests will use a two-sided significance level of 5%.

Primary Objective: To evaluate the safety and reactogenicity of three different dose concentrations of *Na*-APR-1 (M74)/Alhydrogel® co-administered with *Na*-GST-1/Alhydrogel® in healthy Gabonese children.

AEs will be coded according to Medical Dictionary of Regulatory Activities (MedDRA™) preferred terms. The frequency, severity, and relationship of AEs per each dose of *Na*-APR-1 (M74)/Alhydrogel® and *Na*-GST-1/Alhydrogel® compared to Hepatitis B vaccine will be presented in tabular form using the MedDRA™ coded term and organized by MedDRA™ System, Organ, and Class (SOC) designations.

- The frequency of immediate, systemic, and local injection site AEs will be summarized by SOC and preferred term.
- Line listings of clinical and laboratory AEs classified as immediate (within the first 60 minutes), systemic, and local will be displayed in tables stratified by vaccine (i.e., hookworm vs. Hepatitis B vaccines) dose of *Na*-APR-1 (M74)/Alhydrogel® and *Na*-GST-1/Alhydrogel®.
- AEs will be summarized by severity and relationship to vaccine by individuals and dose of *Na*-GST-1 and *Na*-APR-1 (M74).
- The frequency of vaccine-related SAEs will be tallied as well as summarized by body system, by vaccine and dose.

Primary Outcome Measures:

The following summary parameters will be evaluated for each vaccine (hookworm *vs*. Hepatitis B) and dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. Frequency of solicited injection site and systemic reactogenicity, graded by severity, on the day of each study vaccination through 14 days after each study vaccination.
2. Frequency of study vaccine-related serious adverse events from the time of the first study vaccination through approximately 9 months after the last study vaccination.
3. Frequency of clinical safety laboratory adverse events.
4. Frequency of unsolicited adverse events, graded by severity, from the time of each study vaccination through approximately 1 month after each study vaccination.
5. Frequency of new-onset chronic medical conditions through approximately 9 months after the third study vaccination.
6. Frequency of Adverse Events of Special Interest through approximately 9 months after the third study vaccination.

Analysis Plan:

a. The proportion of participants with at least one injection site AE will be compared by vaccine and dose cohort. We will test the null hypotheses that the type and number of adverse events is the same across all groups by Fisher's exact test.

b. Laboratory results (hematological and clinical chemistry) will be examined for trends over time and any clinically significant values for individuals will be reported.

Secondary Objective: To determine the doses of co-administered *Na*-APR-1 (M74) and *Na*-GST-1 that result in the highest levels of IgG antibody approximately 14 days after the third vaccination.

Secondary Outcome Measure:

The following parameters will be evaluated for each dose of *Na*-APR-1 (M74) co-administered with *Na*-GST-1:

1. The IgG level by a qualified indirect ELISA approximately 14 days after the third vaccination.

Analysis Plan:

a. The proportion of participants with detectable anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) responses will be summarized as a descriptive measure.
b. IgG levels will be displayed graphically by study group using notched box plots.
c. Geometric mean antibody responses will be compared between vaccine formulation and dose groups. Comparisons between groups will be made by a one-way analysis of variance (ANOVA) with pair-wise comparisons between groups made by contrasts.
d. Geometric mean antibody responses for each antigen will be compared using a regression model with main effects for the doses of *Na*-APR-1 (10, 30 or 100µg), and *Na*-GST-1 (10, 30 or 100µg).

Tertiary Objective 1: To assess and compare the duration of antibody responses to *Na*-GST-1 and *Na*-APR-1 (M74).

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Tertiary Outcome Measure 1: The IgG antibody response, by indirect enzyme-linked immunosorbent assay (ELISA) at approximately 7, 14, and 28 days after each vaccination, and approximately 3 and 6 months after the third dose.

Analysis Plan:

a. The proportion of participants with detectable anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) responses will be summarized as a descriptive measure.
b. Antigen-specific IgG levels will be displayed graphically by study group using notched box plots at each time point.
c. Geometric mean antibody responses will be compared between vaccine and dose groups at each time point. Comparisons between groups will be made by a one-way

analysis of variance (ANOVA) with pair-wise comparisons between groups made by contrasts.

d. Percent change in IgG antibody levels from days of vaccination to days 7,14 and 28 will be compared between vaccine formulation and dose groups. Values will be reported with 95% confidence intervals.
e. Percent change in IgG antibody levels from vaccination day #1 to #2, and from vaccination day #2 to #3 will be compared between vaccine formulation and dose groups to assess the sustainability of the response from trough to trough. Values will be reported with 95% confidence intervals.
f. Percent change in IgG antibody levels from the peak following the third vaccination, to 3 and 6 months after the third vaccination. Values will be reported with 95% confidence intervals.
g. Geometric mean antibody responses for each antigen will be compared using a regression model at each time point with main effects for the doses of *Na*-APR-1 (M74) (10, 30 or 100µg) and *Na*-GST-1 (10, 30 or 100µg)
h. A longitudinal model will be built to describe the IgG levels over time. Using a longitudinal panel model, differences in antibody isotype levels by dose of *Na*-APR-1 (M74) and *Na*-GST-1 will be explored. This will take account of the correlation between measurements on the same participant.

Tertiary Objective 2: To assess the distribution of IgG subclass responses to *Na*-APR-1 (M74) and *Na*-GST-1.

Tertiary Outcome Measure 2: The IgG subclass distribution (IgG1, IgG3, and IgG4) by indirect ELISA approximately 14 days after the third vaccination.

Tertiary Outcome Measure 3: The IgG1-4 subclass distribution by ELISA on each day of vaccination, approximately 28 days later, and approximately 3 and 6 months after the final vaccination.

Analysis Plan:

a. The proportion of subjects with detectable anti-*Na*-GST-1 and anti-*Na*-APR-1 (M74) IgG1-4 subclass responses will be summarized at each time point as a descriptive measure.
b. IgG1-4 subclass antibody levels will be displayed graphically by study group using notched box plots at each time point.
c. Geometric mean antibody responses will be compared between vaccine and dose groups. Comparisons between groups will be made by a one-way analysis of variance (ANOVA) with pair-wise comparisons between groups made by contrasts.
d. A longitudinal model will be built to describe each of the antibody isotype levels over time for each of IgG1-IgG4. Using a longitudinal panel model, differences in antibody isotype levels by dose of *Na*-APR-1 (M74) and *Na*-GST-1 will be explored. This will take account of the correlation between measurements on the same participant.

Exploratory Objective 1: To assess the cellular immune responses to the *Na*-GST-1 and *Na*-APR-1 (M74) antigens following vaccination.

The following assays will be performed to assess the cellular immune responses to vaccination with *Na*-APR-1 (M74) +/- *Na*-GST-1:

a. Lymphocyte proliferative responses to *in vitro* stimulation with *Na*-GST-1 or *Na*-APR-1 (M74).
b. Cytokine and chemokine production *in vitro* in response to stimulation with *Na*-GST-1 or *Na*-APR-1 (M74).
c. Changes in *in vivo* PBMC subpopulations as determined by flow cytometry.
d. A longitudinal panel analysis will examine for trends over time.

Exploratory Objective 2: To assess the impact of co-administered *Na*-GST-1 and *Na*-APR-1 (M74) on the production of memory B cells specific for each antigen.

a. The percentage of specific memory B cells will be displayed graphically by study group at each time point.
b. Two separate longitudinal panel analyses will test the null hypothesis that average percentages for each of memory B cells are the same in groups over time. This will take account of the correlation between measurements on the same participant.
c. Mann-Whitney tests will assess the null hypothesis that cytokine levels are the same in the groups at the primary time point two weeks after final vaccination.

Exploratory Objective 3: To assess the production of memory B cells specific for each vaccine antigen compared to metabolomic changes before and after vaccination.

Exploratory studies will be performed of changes in the metabolomic profiles in response to vaccination using urine samples that will be analyzed using LC-MS. Data will be analyzed using multivariate modeling and multilevel data analysis pipeline.

Should the study be terminated early, the investigative team will discuss with the SMC the reason for termination and determine which study questions can be addressed in an unbiased manner with the available data. The available data will be analyzed and interpreted in light of early termination.

11 SOURCE DOCUMENTS AND ACCESS TO SOURCE DATA/DOCUMENTS

Complete source documentation (clinical evaluations and test results) will be collected for every child participant for the duration of the study, with supplementary documents (laboratory test reports, supplementary hospital or medical records, etc.) forming part of the source documentation. Case Report Forms (CRFs) will be used to record study-specific data for enrolled subjects, and study-specific data may be entered directly onto CRFs: in these cases, the documents will be both source and CRF. The Principal Investigator will responsible for the accuracy and completeness of the data reported in the CRFs and the source documents. Data reported in the CRFs that is derived from source documents should be consistent with source documents and the discrepancies should be explained.

12 QUALITY CONTROL AND QUALITY ASSURANCE

The investigational site is responsible for conducting routine quality assurance and quality control activities to internally monitor study progress and protocol compliance. The PI will provide direct access to all trial-related sites, source data/documents, and reports for the purpose of monitoring and auditing by the sponsor, and inspection by local and regulatory authorities. The PI will ensure all study personnel are appropriately trained and applicable documentation is maintained on site.

Clinical monitors will verify that the clinical trial is conducted and data are generated, documented (recorded), and reported in compliance with the protocol, ICH/GCP guidelines, and the applicable regulatory requirements. Clinical monitoring reports will be submitted to the Sponsor.

The data management vendor will implement quality control procedures beginning with the data entry system and generate data quality control checks that will be run on the database. Any missing data or data anomalies will be communicated to the site(s) for clarification and resolution.

13 ETHICS/PROTECTION OF HUMAN SUBJECTS

The study will be conducted according to fundamental ethical principles including: The principle of respect for human dignity and the principles of non-exploitation, non-discrimination and non-instrumentalisation. The principle of individual autonomy (entailing the giving of free and informed consent, and respect for privacy and confidentiality of personal data); The principle of justice (the equitable distribution of burdens and benefits of research). The principle of beneficence and non-maleficence, namely with regard to the improvement and protection of health; and, the principle of proportionality (including that research methods are necessary to the aims pursued and that no alternative more acceptable methods are available).

13.1 Ethical Standards

The study will be conducted according to: the Declaration of Helsinki (amended in 2008); CIOMS (Council for International Organizations of Medical Sciences) International Ethical Guidelines for Biomedical Research Involving Human Subjects (2002); International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) (1996) Guideline for good clinical practice E6 (R1); and, the US Code of Federal Regulations (Protection of Human Subjects [21 CFR 50], Institutional Review Boards [21 CFR 56], and Obligations of Clinical Investigators [21 CFR 312]).

13.2 Institutional Review Boards

The investigators will be responsible for obtaining full IRB approvals for the study from the local and national IRB in Gabon. Before the start of the study, the appropriate documents (including the protocol, Investigator's Brochures, and informed consent form) will be submitted to the IRBs. The IRBs will be informed by the Investigator of any new information that may adversely affect the safety of the subjects or the conduct of the study, an annual update and/or request for re-approval, and when the study has been completed.

13.3 Informed Consent and Assent Process

The principles of informed consent in the current edition of the Declaration of Helsinki will be implemented and informed consent obtained in accordance with ICH guidelines. A community meeting will be held to provide volunteers with an overview of the clinical trial and study procedures, and to answer questions in an open forum. This group meeting will be followed by individual informed consent sessions with each interested parent or legal guardian of a potential child participant. During the individual session a member of the study team will read the consent form together with the parent/guardian, regardless of whether or not the parent/guardian is illiterate. The study team member will explain why the parent/guardian's child is being invited to participate in the study and will clarify all of the parent/guardian's questions. Throughout the informed consent process, parents/guardians will be encouraged to ask questions, and if they wish, to discuss the study with family or community members prior to making a decision.

Parents/guardians agreeing to have their child participate in the study will first provide written informed consent and take a true/false comprehension questionnaire. The questionnaire is administered orally in the case of illiterate individuals, and for both written and oral questionnaires study staff will use incorrect answers to identify aspects of the study that require clarification and focus on those areas of the informed consent form for further review with the parent/guardian. All questionnaire questions must be answered correctly, and the informed consent form signed, prior to study screening and enrollment of the child. Parents/guardians unable to read will place an imprint of their finger in the place of a signature; in addition, an independent witness, who is not a member of the study team, will sign the consent form to attest that the informed consent form was read to the parent/guardian, the parent/guardian's questions were answered, and the parent/guardian answered all comprehension questions correctly. The original signed informed consent form for each parent/guardian will be maintained as part of their child's study records. A copy of the informed consent form will be provided to every child's parent/guardian.

The child assent form will also be introduced to the participants because it is directly addressed to the child where procedures related to the study are explained to them in simple language. The child will have to assent and sign. However, if the child is unable to read, an independent witness, who is not a member of the study team will sign the form.

13.4 Subject Confidentiality

All study-related information will be stored securely at the study site. All information on participating children will be stored in locked file cabinets in areas with access limited to study staff. All laboratory specimens, reports, study data collection, and administrative forms will be identified by coded numbers only, to maintain subject confidentiality. All computer entries will also be made using coded number only, and all local databases will be secured with password-protected access systems. Forms, lists, and any other listings that link subject identification numbers to other identifying information will be stored in a separate, locked file in an area with limited access.

Subject study information will not be released without the written permission of the subject, except as necessary for monitoring by the sponsor and/or its designee, the FDA or Gabonese regulatory authorities.

13.5 Study Discontinuation

If the trial is discontinued, children whose parent/guardian signed the informed consent and child assent forms, and are randomized and vaccinated will continue to be followed for safety assessments. No further study vaccinations will be administered.

13.6 Future Use of Stored Specimens

Some of the biological samples collected from study participants may be stored at the local site or at the MITM at GWU or at LUMC in Leiden. Stored samples may be shared with other investigators at other institutions for the purposes of conducting the tests outlined in this study

protocol. Stored samples will not be sold or used directly for production of any commercial product. No human genetic tests will be performed on samples. Each sample will be encoded (labeled) only with a unique code and a unique tracking number to protect subject's confidentiality. There are no benefits to subjects in the collection, storage and subsequent research use of samples. Reports about research done with subject's samples will NOT be kept in their health records. Any proposed future research using samples collected from study participants will first be submitted to the applicable IRBs for approval. The IRBs will determine if the study participants must be re-consented for the use of their samples in such research.

14 DATA HANDLING AND RECORD KEEPING

14.1 Data Management Responsibilities

All data collection forms and laboratory reports must be reviewed by the clinical team and data entry personnel, who will ensure that they are accurate and complete. Adverse events must be recorded on the appropriate data collection form, assessed for severity and relationship to vaccination, and reviewed by the Principal Investigator or appropriate co-investigator.

Data collection is the responsibility of the study personnel at the study site under the supervision of the Principal Investigator. During the study, the Principal Investigator must maintain complete and accurate documentation for the study.

The data management vendor for this study will be responsible for data management, quality review, analysis, and reporting of the study data.

14.2 Data Capture Methods

CRFs will be used to record study-specific data for enrolled subjects, and study-specific data may be entered directly onto CRFs: in these cases, the documents will be both source and CRF. The Principal Investigator will responsible for the accuracy and completeness of the data reported in the CRFs and the source documents. Data reported in the CRFs that are derived from source documents should be consistent with source documents and the discrepancies should be explained.

The information collected on the paper CRFs is entered into an electronic data collection system (EDC); the eCRF. The eCRF will be developed by the data management center (AIGHD). All users who have access to the EDC system will be trained. The access to the eCRF is password controlled. Plausibility checks will be performed according to a data validation plan. Inconsistencies in the data will be queried to the investigators via the electronic data collection system; answers to queries or changes of the data will directly be documented in the system. After all data are entered and all queries are solved, the database will be closed.

14.3 Types of Data

Data for this study will include clinical, safety, and outcome measures (e.g., clinical laboratory values, reactogenicity, and immunogenicity data).

14.4 Timing/Reports

In addition to the study-related documentation required by the regulatory authorities, 4 study reports will be generated. These include 2 interim SMC safety reports, 1 interim safety and immunogenicity study report, and the final study report. The first interim SMC safety report will be completed after the safety data from the Day 14 visits of Group 1 have been compiled, for the purposes of deciding whether it is safe to precede with enrollment into Group 2 of the study. The

second interim SMC safety report will be completed after the safety data from the Day 14 visits of Group 2 have been compiled, for the purposes of deciding whether it is safe to precede with enrollment into Group 3 of the study. The interim study report will be compiled after interim safety and primary immunogenicity data (i.e., antigen-specific IgG responses to *Na*-GST-1 and *Na*-APR-1 (M74) as determined by ELISA) from all Day 208 visits is available and the study investigators unblinded. This report will serve as the basis for deciding whether to continue with future Phase 2 testing of the vaccines in children. A final report containing all safety and immunology data will be prepared after trial completion.

14.5 Study Records Retention

Trial-related documents will be maintained by the Investigator for a period of 2 years after final marketing approval of the vaccine, or if 2 years have elapsed since the formal discontinuation of clinical development of the product. During the study and while archived at the site all study-related information will be stored securely in locked file cabinets in areas with access limited to study staff. All laboratory specimens, reports, study data collection, and administrative forms will be identified by coded number only, to maintain subject confidentiality. All computer entries will be done using a coded number only, and all local databases will be secured with password-protected access systems. Forms, lists, and any other listings that link subject ID numbers to other identifying information will be stored in a separate, locked file in an area with limited access. Subject study information will not be released without the written permission of the subject, except as necessary for monitoring, and the FDA or Gabonese regulatory authorities.

14.6 Protocol Deviations

No modifications to this protocol will be implemented without prior written IRB approval. This does not apply to changes made to reduce discomfort or avert risk to study subjects. Furthermore, in the event of a medical emergency, the investigators shall perform any medical procedures that are deemed medically appropriate. The Principal Investigator must notify the Sponsor and IRBs of all such occurrences. Any change to the protocol will be submitted to the IRB as a protocol amendment, and changes not affecting risk to subjects may be expedited, as appropriate.

Any deviation from the IRB-approved protocol will be documented, including the date and detailed description of the deviation and all corrective actions taken. For any deviation determined to have potential or known impact on subject safety, a report will be submitted to the IRB according to their guidelines and reporting timelines.

15 PUBLICATION POLICY

It is anticipated that results from this study will be published in peer-reviewed journals. If publication is sought, the identity of study subjects or any easily traceable identifiers will not be revealed. Authorship issues will be discussed and agreed upon between the Sponsor and collaborating partners prior to submission for publication. Additionally, the results of the study will be communicated to both study subjects and the community at large.

The Principal Investigator and all partners on this study will make publicly available any final research data resulting from the trial, in a timely fashion following closure of the clinical trial (not more than 12 months after the last subject follow-up visit).

16 LITERATURE REFERENCES

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SUPPLEMENTS/APPENDICES

APPENDIX A1: SCHEDULE OF EVENTS FOR GROUPS 1A, 2A, 3A

Procedures	Blood Volume	Study Days																				
		Pre [1]	0	1	3 ± 1	7 ±1	14 ±2	28 ±4	56 ±7	57	59 ±1	63 ±1	70 ±2	84 ±4	112 ±14	113	115 ±1	119 ±1	126 ±2	140 ±4	200 ±14	290 ±14	380 ±21
Obtain Informed Consent		X																					
Complete History/Physical		X																					
Interim Clinical Evaluation			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AE/SAE/AESI Assessment			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Urine Dipstick + Parasitology		X																					
HCV	1.5 mL	X																					
HBsAg		X																					
HIV		X																					
ALT	1.2 mL	X	X				X		X				X		X				X				
Creatinine		X	X				X		X				X		X				X				
CBC[2]	1.2 mL	X	X				X		X				X		X				X				
Antibody Assays (serum or plasma)	1.2 mL		X			X	X	X	X			X	X	X	X			X	X	X	X	X	
Cellular Assays	5 mL		X			X	X	X	X			X	X	X	X			X	X	X	X	X	
Daily Blood Volume (mL)		3.9	8.6			2.5	8.6	6.2	8.6			2.5	8.6	6.2	8.6			2.5	8.6	6.2	6.2	6.2	
Total Blood Volume (mL)		3.9	12.5			15	23.6	29.8	38.4			40.9	49.5	55.7	64.3			66.8	75.4	81.6	87.8	94	**94**
Urine sample for metabolomics		X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	
Stool Examination		X																		X			X
VACCINATIONS			**1**						**2**						**3**								

[1] Completed within 90 days of first vaccination
[2] CBC safety parameters include WBC, absolute neutrophil count, hemoglobin, and platelet count

APPENDIX A2: SCHEDULE OF EVENTS FOR GROUPS 1B, 2B, 3B

Procedures	Blood Volume	Study Days																				
		Pre [1]	0	1	3 ± 1	7 ±1	14 ±2	28 ±4	56 ±7	57	59 ±1	63 ±1	70 ±2	84 ±4	180 ±14	181	183 ±1	187 ±1	194 ±2	208 ±4	268 ±14	358 ±14	448 ±21
Obtain Informed Consent		X																					
Complete History/Physical		X																					
Interim Clinical Evaluation			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
AE/SAE/AESI Assessment			X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Urine Dipstick + Parasitology		X																					
HCV	1.5 mL	X																					
HBsAg		X																					
HIV		X																					
ALT	1.2 mL	X	X				X		X				X		X				X				
Creatinine		X	X				X		X				X		X				X				
CBC [2]	1.2 mL	X	X				X		X				X		X				X				
Antibody Assays (serum or plasma)	1.2 mL		X			X	X	X	X			X	X	X	X			X	X	X	X	X	
Cellular Assays	5 mL		X			X	X	X	X			X	X	X	X			X	X	X	X	X	
Daily Blood Volume (mL)		3.9	8.6			2.5	8.6	6.2	8.6			2.5	8.6	6.2	8.6			2.5	8.6	6.2	6.2	6.2	
Total Blood Volume (mL)		3.9	12.5			15	23.6	29.8	38.4			40.9	49.5	55.7	64.3			66.8	75.4	81.6	87.8	94	**94**
Urine sample for metabolomics		X	X	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	X	X	
Stool Examination		X																		X			X
VACCINATIONS			**1**						**2**						**3**								