PROTOCOL

Study title:

The persistence of vaccine-induced HBsAg-specific antibodies and immune memory in adults after complete primary vaccination with the GlaxoSmithKline (GSK) hexavalent Infanrix vaccine at infancy

Short title:

HEP-IMMUNE

Research institution:

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1. Abbreviations

Anti-HBc	Hepatitis B core antigen specific antibodies
Anti-HBs	Hepatitis B surface antigen specific antibodies
APC	According to protocol cohort
CEV	Centre for the Evaluation of Vaccination
CMI	Cell mediated immunity
DTPa	Diphtheria-tetanus-acellular pertussis
eCRF	Electronic case report form
ELISA	Enzyme-linked immunosorbent assay
ELISPOT	Enzyme-linked immunosorbent spot
FAMPH	Federal Agency for Medicines and Health products (Belgium)
GMC	Geometric mean concentration
HBc	Hepatitis B core antigen
HBsAg	Hepatitis B surface antigen
HBV	Hepatitis B vaccine
HIV	Human immunodeficiency virus
IPV	Inactivated polio vaccine
Hib	Haemophilus Influenzae type b vaccine
mIU	Milli-international units
mL	Milliliter
µg	Microgram
PBMC	Peripheral blood mononuclear cell
SAEs	Serious adverse events
TVC	Total vaccinated cohort

2. Introduction – rationale of the study

Long-term persistence of HBsAg-specific antibodies (anti-HBs) and persistence of HBsAg-specific memory B and CD4+ T cells after administration of the monovalent Engerix-B vaccine have been intensively investigated and documented in several clinical trials [1-4]. Infanrix hexa, a hexavalent diphtheria-tetanus-acellular pertussis-hepatitis B-polio-Haemophilus influenzae type b (DTPa-HBV-IPV-Hib) became available on the market in Belgium in 2002. In 2004, it became free of charge and was added to the Belgian recommendation for vaccination of children. Clinical studies indicate that vaccination in infancy with the hexavalent vaccine results in a long-term protection of at least 15 years [5-8]. These studies do however also suggest decreasing seroprotection rates over time, with seroprotection rates of 72.2% and 53.7% in healthy children aged 7-8 years and 12-13 years, respectively [5, 8].

In order to investigate the need of a challenge vaccination to extend protection through adulthood the goal of this observational trial is to determine the persistence of anti-HBs in adults who received a 3-dose or 4-dose schedule with the hexavalent DTPa-HBV-IPV-Hib vaccine (Infanrix hexa) in infancy. In addition, the anamnestic response will be evaluated in all subjects with a titre below the seroprotection level of 10 mIU/mL and who received a single challenge dose (the so-called ‘diagnostic vaccination’) with Engerix-B outside of the study. The

presence of memory B-cells and CD4-positive T-cells will be explored in all subjects who receive a challenge vaccination (pre-challenge and post-challenge) and in a subset of subjects showing a protective level of anti-HBs at baseline (no challenge required) to compare cellular mediated immunity of these subpopulations.

3. Study objectives, endpoints and estimands

3.1 Objectives

The primary objectives of this study are:

- To assess the persistence of immunity to hepatitis B in terms of seroprotection rate (refer to below for a definition) in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy.
- To assess the persistence of immunity to hepatitis B in terms of seroprotection rate, percentage of subjects with an anamnestic response in response to an Engerix-B challenge dose in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy who have an anti-HBs concentration < 10 mIU/mL at the start of the study.

The secondary objectives of this study are:

- To assess the persistence of immunity to hepatitis B in terms of Geometric Mean Concentration (GMC), seropositivity rate, percentage of subjects with anti-HBs antibody concentration ≥ 100 mIU/mL and T cell and memory B cell mediated immune responses to hepatitis B surface antigen in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy.

- To assess the persistence of immunity to hepatitis B in terms of GMC, seropositivity rate, percentage of subjects with anti-HBs antibody concentration ≥ 100 mIU/mL, percentage of subjects with a ≥ 4-fold increase in anti-HBs antibody concentration, T cell and memory B cell mediated immune responses to hepatitis B surface antigen in response to an Engerix-B challenge dose in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy who have an anti-HBs concentration < 10 mIU/mL at the start of the study.

- To evaluate the safety and reactogenicity of Engerix-B challenge dose in terms of serious adverse events (SAEs).

Within the context of this study, we consider a participant as “seroprotected” when anti-HBs antibody concentrations are ≥ 10 mIU/mL, as this is the common definition of seroprotection within the context of hepatitis B studies in medical literature. However, this cut-off does not necessarily mean participants with anti-HBs antibody concentrations < 10 mIU/mL are not protected against hepatitis B. This is one of the reasons for looking at other (cellular) markers of immunity in this study.

3.2 Endpoints

Primary endpoints

- Persistence of immunity to hepatitis B in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy.
    - Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL at the baseline visit.
- Persistence of immunity to hepatitis B in response to an Engerix-B challenge dose in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy who have an anti-HBs concentration < 10 mIU/mL at the start of the study.
    - Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL at Day 8 and Day 29 post-challenge dose time points.
    - Percentage of subjects who respond to the challenge dose at Day 8 and Day 29 post-challenge time points, in which a response is defined as follows:
        - For subjects who are seronegative at the baseline visit: anti-HBs antibody concentrations ≥ 10 mIU/mL at post-challenge dose time points.
        - For subjects who are seropositive at the baseline visit: a ≥ 4-fold increase in anti-HBs antibody concentrations at post-challenge dose time points, compared to the baseline visit.

Secondary endpoints

- Persistence of immunity to hepatitis B in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy.
    - Percentage of subjects with anti-HBs antibody concentrations ≥ 2 mIU/mL and ≥ 100 mIU/mL at the baseline visit.
    - Anti-HBs antibody concentrations at the baseline visit (geometric mean titre).
    - Hepatitis B specific memory B cell-mediated immune responses (frequency of HBs-specific memory B cells) at Day 1, before vaccination.
    - Hepatitis B specific T cell-mediated immune responses (frequency of HBs-specific CD4 T-lymphocytes) at Day 1, before vaccination.
- Persistence of immunity to hepatitis B in response to an Engerix-B challenge dose in adult subjects vaccinated with three or four doses of Infanrix hexa during infancy who have an anti-HBs concentration < 10 mIU/mL at the start of the study.
    - Percentage of subjects with anti-HBs antibody concentrations ≥ 2 mIU/mL and ≥ 100 mIU/mL at Day 8 and Day 29 post-challenge dose time points.
    - Percentage of subjects who are seropositive at the baseline visit and show a ≥ 4-fold increase in anti-HBs antibody concentrations at Day 8 and Day 29 post-challenge dose time points.
    - Anti-HBs antibody concentrations at Day 8 and Day 29 post-challenge dose time points (geometric mean titre).

  - Hepatitis B specific memory B cell-mediated immune responses (frequency of HBs-specific memory B cells) at Day 8 and Day 29 post-challenge dose time points.
  - Hepatitis B specific T cell-mediated immune responses (frequency of HBs-specific CD4 T-lymphocytes) at Day 8 and Day 29 post-challenge dose time-points.
- Serious adverse events
  - Occurrence of serious adverse events between Day 1 and up to study end.
  - Occurrence of serious adverse events related to the challenge dose between Day 1 and up to study end.

Whenever possible, the reporting of endpoints will be conducted for the overall study population, as well as stratified by humoral immunity to hepatitis B:
- subgroup 1: anti-HBs concentration ≥ 10 mIU/mL at baseline
- subgroup 2: anti-HBs concentration < 10 mIU/mL at baseline and anti-HBs concentration ≥ 10 mIU/mL after the Engerix-B challenge dose
- subgroup 3: anti-HBs concentration < 10 mIU/mL at baseline and anti-HBs concentration < 10 mIU/mL after the Engerix-B challenge dose

4. Study methodology

4.1 Study design

The study design is summarized in **Figure 1** and **Table 1**. During the baseline visit (≤28d before the Day 1 visit), healthy adult participants will be included in the study if they meet all inclusion criteria and do not meet any exclusion criteria (refer to **Section 5.1** and **Section 5.2** for an overview of the eligibility criteria). This assessment will be made based on anamnesis, and physical examination (including the measurement of vital signs), if deemed necessary by the investigator. A venous blood draw will be performed to assess the anti-HBs antibody concentration. Anti-HBc antibody concentrations will be determined in parallel and participants with a positive anti-HBc test result will be excluded from further study activities.

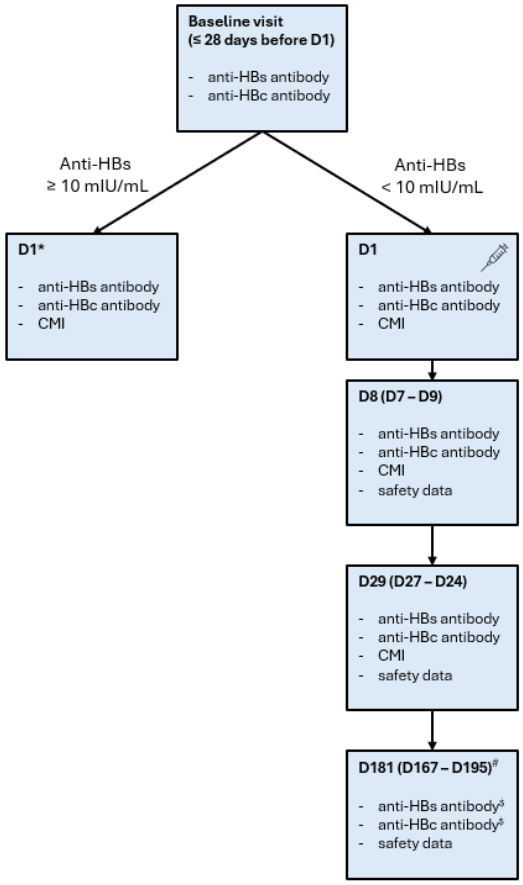


Figure 1: Overview of study activities.
*D1 will be conducted in participants with anti-HBs antibody titers < 10 mIU/mL and in a subset of participants with anti-HBs antibody titers ≥ 10 mIU/mL. Note that on Day 1, a challenge dose with Engerix-B is given (outside the study) to subjects with anti-HBs antibody titers < 10 mIU/mL. CMI samples are collected before this vaccination. # Data at Day 181 is only collected in participants who did not respond to the challenge dose with Engerix-B by Day 29 (anti-HBs antibody titers < 10 mIU/mL). The data for Day 181 will be collected via a phone call. $ All available anti-HBs antibody titers measured between Day 29 and Day 181 will be requested from Mensura and double-checked during the Day 181 phone call with the participant: no additional blood draw is scheduled at the study site. CMI, cellular mediated immunity; D, day; HBc, hepatitis B core antigen; HBs, hepatitis B surface antigen.

Table 1: Schedule of Activities.

Visit	**Baseline visit**	**Day 1[1]**	**Day 8[2]**	**Day 29[2]**	**Day 181[3]**
Timing	Day -28 to Day 1	Day 1	Day 7 to Day 9	Day 27 to Day 34	Day 167 to Day 195
Type of contact	Visit	Visit	Visit	Visit	Phone contact
Informed consent	X				
Collect demographic data	X				
Collect medical history	X	X			
Collect vaccination history	X	X			
Physical examination	X[4]	X[4]	X[4]	X[4]	
Vital signs measurement	X[4]	X[4]	X[4]	X[4]	

Check for pregnancy in WOCBP	X	X			
Check inclusion/exclusion criteria	X	X			
Recording of administered challenge dose[5]		X			
Distribution of participant card	X				
Record concomitant medications and vaccinations		X	X	X	X
Record any intercurrent medical conditions		X	X	X	X
Record serious adverse events		X	X	X	X
Record pregnancies		X	X	X	X
Blood sampling for immunogenicity	X	X	X	X	
Record immunogenicity measurements[6]					X
Study conclusion[7]	X[6]	X[6]		X[6]	X[6]

[1] For participants with anti-HBs antibody titers ≥ 10 mIU/mL during the baseline visit, only a random subset will be invited for the Day 1 visit: they will not receive a challenge dose. All participants with anti-HBs antibody titers < 10 mIU/mL during the baseline visit will be invited for the Day 1 visit, and they will all receive a challenge dose.
[2] These visits will only be conducted in participants that received a challenge dose during the Day 1 visit.
[3] This visit will only be conducted in participants that received a challenge dose during the Day 1 visit and that had anti-HBs antibody titers < 10 mIU/mL during the Day 29 visit.
[4] A physical examination and the measurement of vital signs can be performed if deemed necessary by the investigator.
[5] The administration of the challenge dose is not part of the study procedures, as it is not performed by study site personnel but by Mensura as part of their normal workflow: HEP-IMMUNE remains an observational study.
[6] Mensura will follow up participants that do not respond to the challenge dose. Mensura will provide the study site with additional immunogenicity measurements after these participants have received additional doses of Engerix-B. Measurements collected between the Day 29 and Day 181 visit will be reviewed with the study participants and recorded in the eCRF.
[7] The duration of the study depends on the anti-HBs antibody titers measured during the study as well as the allocation to the immunogenicity subset for participants with anti-HBs antibody titers ≥ 10mIU/mL (refer to [1], [2] and [3] above).
WOCBP, women of child-bearing potential.

From the participants who are considered seroprotected (anti-HBs antibody concentration ≥ 10 mIU/mL) during the baseline visit, a subset of 50 subjects will be randomly selected to come on a Day 1 visit for collection of peripheral blood mononuclear cells (PBMCs) to assess cellular mediated immunity.

All participants who have an anti-HBs concentration < 10 mIU/mL will receive a challenge vaccination on the Day 1 visit (Engerix-B, 1mL of 20µg HBsAg/mL), outside of the study (by Mensura, the company responsible for occupational medicine at the University of Antwerp). Prior to study vaccine administration, contra-indications for further participation in the study will be evaluated by the CEV team. Female subjects of childbearing potential will be asked anamnestically for not being pregnant. Venous blood samples will be collected on Day 1 (pre-challenge), Day 8 and Day 29 visits to assess both humoral immunity (anti-HBs and anti-HBc antibody concentration) and cellular mediated immunity. In addition, serious adverse events will be collected between Day 1 up to the end of the study via interviews with study personnel.

A maximum of approximately 37 mL of blood per participant will be collected per visit (5 mL to assess humoral immunity, 27 mL to assess cellular mediated immunity, and 5 mL as a back-up sample for further research). The total volume of blood collected in the study will be between 10 mL and 116 mL, depending on the subset the participant belongs to (refer to **Table 2**). In event of technical issues, blood collections may be repeated. However, under no circumstances will the total volume of blood collected during the study exceed 250 mL.

Participants who have an anti-HBs concentration < 10 mIU/mL 28 days after the challenge vaccination, will be considered non-responders. These subjects will be advised to follow a non-responder schedule outside of the study (2 doses of Engerix-B simultaneously, followed by 2 additional doses after 2 months). These non-responders will be contacted via phone 6 months after the start of the study to verify whether participants responded to the non-response schedule. No additional blood draw at the study site is foreseen for these participants, but Mensura will provide the additional immunogenicity measurements that they collected between the Day 29 and Day 181 visit.

5. Study population and sample size:

Approximately 250 healthy adults will be enrolled in the study if they meet all of the inclusion criteria and do not meet any of the exclusion criteria. For the rationale behind this sample size and the expected precision, we refer to **Section 9.1**. Of the participants with anti-HBs antibody titers ≥ 10 mIU/mL during the baseline visit, 50 will be included in the immunogenicity subset that will return for the assessment of cell-mediated immunity.

5.1 Inclusion criteria

All subjects have to satisfy all of the following criteria at study entry:

1. A male or female of age greater than or equal to 18 years old.
2. Written informed consent obtained from the subject.
3. Subjects who, in the opinion of the investigator, can and will comply with the requirements of the protocol (e.g., report safety events, return for follow-up visits).
4. Documented evidence of previous vaccination with
   a) three consecutive doses of Infanrix hexa during infancy with
      - an interval of at least 2 months between the 2 primary doses, and
      - the third (challenge) dose given at least 6 months after the last priming dose and given between 11 and 13 months of age, and
      - no subsequent challenge dose ever received later.

      OR

   b) four consecutive doses of Infanrix hexa during infancy with
      - an interval of at least 1 month between the 3 primary doses, and
      - the fourth (challenge) dose given at least 6 months after the last priming dose and given before 18 months of age, and
      - no subsequent booster dose ever received later.
5. Female subjects of non-childbearing potential can be enrolled in the study.
6. Female subjects of childbearing potential can be enrolled in the study if the participant
   - Has practiced adequate contraception for 28 days prior to study start administration, and
   - Has agreed to continue adequate contraception until study end
7. Subject agrees to receive one challenge dose with Engerix-B – outside of the study – if anti-HBs antibody titers are < 10 mIU/mL at the baseline visit.

5.2 Exclusion criteria

The following criteria are checked at the time of study entry. If any exclusion criterion is met, the subject cannot be included in the study:

Medical conditions

1. Any confirmed or suspected immunosuppressive or immunodeficient condition, including HIV infection, based on medical history and physical examination (no laboratory testing required).
2. History of hepatitis B disease or episode of jaundice with unknown etiology.
3. History of any reaction or hypersensitivity likely to be exacerbated by any component of the challenge vaccine.
4. Alcoholism or substance use disorder within the past 2 years, as per the judgment of the investigator.
5. Any other clinical condition that, in the opinion of the investigator, might pose additional risk to the participant due to participation in the study.

Use of medication

6. Administration of an additional hepatitis B challenge vaccination since completion of the primary vaccination series.
7. Planned administration of a vaccine not foreseen by the study protocol within 28 days before study start, or planned administration during the study period.
8. Use of any investigational or non-registered product (drug, vaccine or invasive medical device) during the period starting 28 days before study start, or planned use during the study period.
9. Chronic administration (defined as more than 14 days in total) of immunosuppressants or other immune-modifying drugs during the period starting 3 months prior to study start. For corticosteroids, this will mean prednisone equivalent ≥ 20 mg/day. Inhaled, topical and intraarticular steroids are allowed.
10. Administration of long-acting immune-modifying drugs within 3 months before enrollment or planned use at any time during the study period (e.g., infliximab).
11. Administration of immunoglobulins and/or any blood products or plasma derivatives during the period starting 3 months before study start, or planned administration during the study period.

Other

12. Pregnant or lactating female.
13. Female planning to become pregnant or planning to discontinue contraceptive precautions before end of study.
14. Concurrently participating in another clinical study, at any time during the study period, in which the participant has been or will be exposed to an investigational or a non-investigational intervention (drug/invasive medical device).

6. Study procedures

6.1 Recruitment

This study will be conducted in collaboration with the external Service for occupational prevention and protection at the UA (Mensura). Participants will be recruited from students at the University of Antwerp that are enrolled in the academic programs for the bachelor degree of Biomedical Sciences, Medicine, Pharmaceutical Sciences and Rehabilitation Sciences and Physiotherapy. These students are targeted because they are required to prove seroprotection against hepatitis B (anti-HBs concentration ≥ 10 mIU/mL) before they can start with their

traineeships. At the beginning of the academic year these students are invited by Mensura for HBs titer control and revaccination if necessary. At the same time they will be informed by the CEV team about the study and invited to participate.

All participants who provide written informed consent (ICF) to participate in the study will receive a participant identification number. The first participant will receive identification number 001 and subsequent participants will be assigned sequential numbers. In case of rescreening, participants will retain their participant identification number.

6.2 Clinical procedures

6.2.1 Collection of demographic data and medical history

Demographic data such as age, sex, race and ethnicity are documented in the eCRF. This is necessary to assess the diversity of the study participants.

The medical/vaccination history will be obtained by interviewing the subject and reviewing the participant's medical records. Any pre-existing conditions, signs and symptoms that are present prior to the administration of the study intervention and deemed clinically significant by the investigator are recorded in the eCRF and coded using the Medical Dictionary for Regulatory Activities (MedDRA) dictionary (version of 2026 or later).

6.2.2 Collection of vaccination history and medication use

The study physician will review the vaccination history and use of concomitant medication/vaccinations of the subject at each visit. The following medication and vaccines are recorded in the eCRF:

- All prior vaccines, including investigational vaccines, administered within 28 days prior to signing the informed consent form.
- All prior hepatitis B vaccines, including investigational hepatitis B vaccines.
- All concomitant medication, including vaccines, except vitamins and dietary supplements, that the participant receives at the time of signing the informed consent form up to the end of the study.
- All systemic antibiotics, antiviral medication, anti-inflammatory medication or analgesics used within 48 hours prior to the administration of Engerix-B during the Day 1 visit.

For all concomitant medication, the reason for use, the start and end date of administration of the medication and dosage information will be recorded in the eCRF. Concomitant medication will be coded using the WHO Drug Dictionary (version of 2026 or later).

6.2.3 Physical examination and collection of vital signs

A targeted physical examination and collection of vital signs will be done only if deemed necessary by the investigator.

6.2.4 Collection of blood samples

An overview of the biological samples that will be collected in this study can be found in **Table 2**. Repeat samples may be taken in case of technical issues with the originally collected

samples. The maximum amount of blood collected from each participant over the entire duration of the study will not exceed 250 mL.

Table 2A: Approximate volume of biological samples collected at each timepoint and during the whole study, stratified by participant group[a].

Sample type	Baseline	Day 1	Day 8	Day 29	Total
Seroprotected at baseline (anti-HBs antibody concentration ≥ 10 mIU/mL), non-subset					
Blood sample for humoral immunity	5 mL	-	-	-	10 mL
Blood sample for cellular immunity	-	-	-	-	
Back-up sample	5 mL	-	-	-	
Total	10 mL	-	-	-	
Seroprotected at baseline (anti-HBs antibody concentration ≥ 10 mIU/mL), subset					
Blood sample for humoral immunity	5 mL	5 mL	-	-	42 mL
Blood sample for cellular immunity	-	27 mL	-	-	
Back-up sample	5 mL	-	-	-	
Total	10 mL	32 mL	-	-	
Not seroprotected at baseline (anti-HBs antibody concentration < 10 mIU/mL)					
Blood sample for humoral immunity	5 mL	5 mL	5 mL	5 mL	116 mL
Blood sample for cellular immunity	-	27 mL	27 mL	27 mL	
Back-up sample	5 mL	-	5 mL	5 mL	
Total	10 mL	32 mL	37 mL	37 mL	

[a] In case of repeat sampling, the total volume of blood collected during the study will not exceed 450 mL.

6.2.5 Safety assessment

As Engerix-B is a commercially available vaccine that has been used for more than 30 years, the assessment of reactogenicity will be limited. No solicited or unsolicited adverse events will be collected, with the exception of serious adverse events (SAEs). SAEs will be collected from the signing of the ICF up to the end of study. For the definition of an adverse event and SAE, refer to **Appendix 1**. For each SAE, the severity, outcome and relationship with the administration of Engerix-B will be assessed as described in **Appendix 2**. Information about the occurrence of safety events will be collected via interviews with the study personnel during study visits or phone calls.

All SAEs will be recorded in the eCRF and reported by the investigator to the Sponsor and GSK within 24 hours of awareness. At the time of the initial report, the investigator must provide an assessment of causality. All require follow-up until resolution, stabilization, the event is otherwise explained, or the participant is lost to follow-up. The investigator is required to proactively follow each participant after the initial SAE report.

Details of all pregnancies in participants that begin between Day 1 and up to the end of the study are collected and a pregnancy report form should be submitted to the Sponsor within 24 hours of awareness. Pregnancies in partners of study participants do not require reporting. The Sponsor will report pregnancy information on any female study participant who becomes pregnant while participating in the Study and following exposure to a GSK Product, to GSK (as

specified below) using an approved form within 24 hours of first becoming aware of the pregnancy. The participant will also be followed to determine the outcome of the pregnancy (including any premature termination of the pregnancy). Information on the status of the mother and child will be forwarded to GSK. Generally, follow-up will be requested by GSK no longer than 6 to 8 weeks following the estimated delivery date. Study participants who become pregnant are asked to sign a separate ICF for the follow-up of pregnancy.

Intercurrent medical conditions will be collected from Day 1 up to the end of study. Intercurrent medical conditions are conditions that could influence immunogenicity (e.g. either a confirmed immunodeficiency condition, new malignancy, or development of hepatitis B).

6.3 Screen failures

A participant who has consented to participate in the study but does not meet all inclusion criteria and/or meets exclusion criteria is considered a screen failure. To ensure transparent reporting of screen failure participants and to meet the Consolidated Standards of Reporting Trials (CONSORT) publishing requirements, a limited amount of information of screen failure participants is retained. In particular, the informed consent date, demographic information and the eligibility criteria are reported in the eCRF.

Individuals who do not meet the eligibility criteria may be rescreened. Rescreened participants retain the same participant identification number and do not have to sign a new informed consent form.

6.4 Participant discontinuation/withdrawal

6.4.1 Participant discontinuation/withdrawal

Participants may withdraw from the study at any time at the participant's own request for any reason. A participant is not obligated to provide a reason for his/her withdrawal from the study. Investigators will attempt to contact participants who do not return for scheduled visits or follow-up.

A participant may be withdrawn from the study at any time by the investigator for safety, behavioural or compliance reasons. If a subject is withdrawn from further vaccine administration due to safety reasons, the investigator is required to proactively follow each participant at subsequent visits /contacts.

All data and samples collected up to the date and time of withdrawal or last contact with the participant will be included in the study analyses. If the participant withdraws consent for disclosure of future information, the Sponsor may retain and continue to use any data collected before such a withdrawal of consent.

If a participant withdraws from the study, the participant may request the destruction of any samples taken and not yet tested, and the investigator will document this in the study site records. A participant can also request for the destruction of the samples any time during the study. This request will be documented in the study site records.

The primary reason for participant discontinuation/withdrawal from the study will be documented in the eCRF based on one of the reasons documented in **Table 3**.

Table 3: Reason for participant discontinuation/withdrawal from the study.

Reasons	Additional items/sub-reasons
Adverse Event	Specify
Lost to follow-up	
Withdrawal of consent	
Investigator decision	Specify
Protocol deviation	Specify
Study terminated by Sponsor	
Death	
Other	Specify

6.4.2 Lost to follow-up

A participant is considered lost to follow-up if the participant does not return to scheduled visits and cannot be reached by the study site.

If a participant fails to return to a scheduled study visit, the study site will attempt to contact the participant by phone, e-mail and or certified letter to the participant's last known mailing address. At least 3 documented attempts will be made before a participant is considered lost to follow-up.

Participants who are lost to follow-up will be considered as withdrawn from the study and will be documented as such in the eCRF.

6.5 Laboratory procedures

6.5.1 Assessment of immunogenicity

Humoral immunity samples will be sent to Centrum voor Medische Analyses (CMA) laboratory, Herentals for analyses of HBs and HBc antibody titers. Samples for cellular mediated immunity will be analysed by the Laboratory of Experimental Haematology, Vaccine & Infectious Disease Institute (VAXINFECTIO). Further details are explained in the laboratory manual.

Serological assays for the determination of anti-HBs and anti-HBc antibody concentrations will be performed by enzyme-linked immunosorbent assay (ELISA) at a laboratory using standardized and validated procedures. Seropositivity and seroprotection will be defined as an anti-HBs antibody concentration above the lower limit of quantification (2 mIU/mL) and ≥ 10 mIU/mL, respectively.

To assess cellular mediated immunity, whole blood cell samples will be collected. PBMC separation will be performed within 24 hours of blood collection. Frequencies of memory B-cells secreting anti-HBs will be measured using a B-cell enzyme-linked immunosorbent spot (ELISPOT) assay. Additionally, frequencies of HBsAg-specific CD4-positive T-cells expressing activation markers (CD40 ligand, interleukin-2, interferon gamma and tumour necrosis factor alpha) will be measured by intracellular cytokine staining and cytokine flow cytometry on PBMCs stimulated with HBsAg.

6.5.2 Long term sample storage

Samples for future research will be stored at the Biobank of the University of Antwerp for a maximum of 15 years.

6.6 Compensation

Subjects will receive a compensation fee for their time and transportation costs of €50 per visit per scheduled study visit (with a maximum of 4 visits). Non-responders who followed a non-response schedule outside the study will receive an additional sum of €10 for the phone contact 6 months after study start. The total sum will be transferred to the bank account of the participant according to the practical arrangements of the study site.

7. Data management

Study data will be entered into the electronic Case Report Form (eCRF) using REDCap electronic data capture system in compliance with ICH E6. Each study subject will be only identified in the eCRF by a unique numeric code to ensure subject confidentiality (the participant identification number).

8. Archiving

Access to the eCRF or source documents will be restricted to the researchers only. The electronic database will be stored in a password protected centralized database server and source documents will be kept in locked data room at the offices of the CEV. No personal identifying information will be disclosed to anyone outside the research team. The source documents as well as the electronic database will be stored securely for the period of 25 years after completion of the study and will remain available for internal audits and/or inspections of regulatory authorities.

9. Statistical analysis

9.1 Sample size calculation

To calculate the required sample size, the percentage of participants with anti-HBs antibody concentrations ≥ 10 mIU/mL was identified as the main estimated parameter of interest. Based on available medical literature [5-8], the expected value of this parameter is 50% (**Table 4**). To achieve an absolute margin of error of 5% and a confidence level of 95%, a sample size of 402 subjects would be required. A more feasible sample size of ~250 participants would allow us to estimate the parameter estimate with an absolute margin of error of 6-7%, assuming that about 10% of the required datapoints are not available for the analysis of the primary endpoint. This margin of error was deemed acceptable.

Table 4: Reference values in medical literature.

Study	Country	Population (age in years)	N	Anti-HBs antibody concentration (% [95% CI])		
				≥ 6.2 mIU/mL	≥ 10 mIU/mL	≥ 100 mIU/mL
Van den Meeren et al [5]	Germany	7 – 8	284	78.5 % (73.3; 83.2)	72.2 % (66.6; 77.3)	30.6 (25.3; 36.4)
Avdicova et al [6]	Slovakia	11 – 12	95	55.8 % (45.2; 66.0)	48.4 % (38.0; 58.9)	14.7 % (8.3; 23.5)
Behre et al [7]	Germany	12 – 13	291	69.8 % (64.1; 75.0)	60.5 % (54.6; 66.1)	21.0 % (16.4; 26.1)
Schwarz et al [8]	Germany	14 – 15	268	60.8 % (54.7; 66.7)	53.7 % (47.6; 59.8)	16.8 % (12.8; 19.1)

N = number of subjects in the baseline cohort for immunogenicity
Confidence intervals are 95% exact confidence intervals
Seroprotection is defined as anti-HBs antibody concentration ≥ 10 mIU/mL

The required sample size to achieve other margin or errors for different expected percentages of participants with anti-HBs antibody concentrations ≥ 10 mIU/mL are denoted in **Table 5**.

Table 5: Required sample sizes for different margin of errors and expected percentage of participants with anti-HBs antibody concentrations ≥ 10 mIU/mL. *Margin of errors that can be achieved with a sample size of 250 are denoted in green.*

Margin of error	Expected percentage of participants with anti-HBs antibody concentrations ≥ 10 mIU/mL							
	35%	40%	45%	50%	55%	60%	65%	70%
5%	367	386	398	402	398	386	367	341
6%	257	271	279	281	279	271	257	238
7%	190	200	206	208	206	200	190	177
8%	147	155	159	160	159	155	147	137
9%	117	123	126	127	126	123	117	108
10%	95	100	103	104	103	100	95	88
15%	43	45	47	47	47	45	43	40
20%	24	26	27	27	27	26	24	23

9.2 Definition of analysis sets

1. Total Vaccinated Cohort (TVC)

- The TVC will include all subjects who receive the Engerix-B challenge dose.

The TVC will be used for the analysis of the safety endpoint.

2. According to Protocol Cohort (APC)

- The APC will include all subjects who satisfy all of the following criteria

  a) subjects who meet all eligibility criteria

  b) subjects who comply with the procedures and visit intervals as defined in the study protocol

  c) subjects for whom immunogenicity results are available

The APC will be used for the analyses of all immunogenicity endpoints.

9.3 Statistical methods to analyse the primary endpoints

For primary endpoint 1, the following analyses will be conducted on the APC:

- Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL at the baseline visit will be calculated, with exact 95% confidence intervals.
- The distribution of anti-HBs antibody concentrations will be displayed using a reverse cumulative curve.

For primary endpoint 2, the following analyses will be conducted on the APC:

- Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL and ≥ 100 mIU/mL at the Day 8 visit and Day 29 visit, with exact 95% confidence intervals.
- Percentage of subjects who repons to the challenge dose at Day 8 and Day 29 post-challenge time points, with exact 95% confidence intervals, in which a response is defined as follows:

  - For subjects who are seronegative at the baseline visit: anti-HBs antibody concentrations ≥ 10 mIU/mL at post-challenge dose time points.
  - For subjects who are seropositive at the baseline visit: a 4-fold increase in anti-HBs antibody concentrations at post-challenge dose time points, compared to the baseline visit.
- The distribution of anti-HBs antibody concentrations at Day 8 and Day 29 post-challenge time points will be displayed using a reverse cumulative curve for the Day 8 visit and Day 29 visit.

For primary endpoint 3, the following analyses will be conducted on the APC:

- Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL at the baseline visit or with an anti-HBs concentration ≥ 10 mIU/mL at the Day 29 post-challenge dose time point, with exact 95% confidence intervals.
- Percentage of subjects with anti-HBs antibody concentrations ≥ 10 mIU/mL at the baseline visit or with an anti-HBs concentration ≥ 10 mIU/mL at the Day 29 post-challenge dose time point, with exact 95% confidence intervals.

9.4 Statistical methods to analyse the secondary endpoints

For secondary endpoint 1, the following analyses will be conducted on the APC:

- Percentage of subjects with anti-HBs antibody concentrations ≥ 2 mIU/mL and ≥ 100 mIU/mL at the baseline visit will be calculated, with exact 95% confidence intervals.
- Geometric mean concentrations with 95% confidence intervals will be calculated for anti-HBs antibody concentrations at the baseline visit.
- The relationship between the pre-challenge anti-Hbs concentration and Day 29 post-challenge anti-Hbs concentration will be presented by a regression line graph.
- The frequency of Hepatitis B specific memory B cells at the Day 1 visit will be evaluated by calculating the geometric mean frequency with a 95% confidence interval, the median, and the interquartile range. The analysis will be stratified by the baseline anti-Hbs antibody concentrations.
- The frequency of Hepatitis B specific memory B cells at the Day 1 visit will be visualized using boxplots, stratified by the baseline anti-Hbs antibody concentrations.
- The frequency of Hepatitis B specific CD4 T-lymphocytes at the Day 1 visit will be evaluated by calculating the geometric mean frequency with a 95% confidence interval, the median, and the interquartile range. The analysis will be stratified by the initial anti-Hbs antibody concentrations.
- The frequency of Hepatitis B specific CD4 T-lymphocytes at the Day 1 visit will be visualized using boxplots, stratified by the baseline anti-Hbs antibody concentrations.

For secondary endpoint 2, the following analyses will be conducted on the APC:

- Percentage of subjects with anti-HBs antibody concentrations ≥ 2 mIU/mL at the Day 8 visit and Day 29 visit, with exact 95% confidence intervals.
- Geometric mean concentrations with 95% confidence intervals will be calculated for anti-HBs antibody concentrations at the Day 8 visit and Day 29 visit.
- The frequency of Hepatitis B specific memory B cells at the Day 8 visit and Day 29 visit will be evaluated by calculating the geometric mean frequency with a 95% confidence interval, the median, and the interquartile range.

- The frequency of Hepatitis B specific memory B cells at the Day 8 visit and Day 29 visit will be visualized using boxplots.
- The relationship between the pre-challenge Hepatitis B specific memory B cells and Day 30 post-challenge Hepatitis B specific memory B cells will be presented by a regression line graph.
- The frequency of Hepatitis B specific CD4 T-lymphocytes at the Day 8 visit and Day 29 visit will be evaluated by calculating the geometric mean frequency with a 95% confidence interval, the median, and the interquartile range.
- The frequency of Hepatitis B specific CD4 T-lymphocytes at the Day 8 visit and Day 29 visit will be visualized using boxplots.
- The relationship between the pre-challenge Hepatitis B specific CD4 T-lymphocytes and Day 30 post-challenge Hepatitis B specific CD4 T-lymphocytes will be presented by a regression line graph.

For secondary endpoint 3, the following analyses will be conducted on the TVC:

- The occurrence of serious adverse events related to the challenge dose, up to study end, will be described in detail.

Please note that, whenever possible, the reporting of endpoints will be conducted for the overall study population, as well as stratified by humoral immunity to hepatitis B:
- subgroup 1: anti-HBs concentration ≥ 10 mIU/mL at baseline
- subgroup 2: anti-HBs concentration < 10 mIU/mL at baseline and anti-HBs concentration ≥ 10 mIU/mL after the Engerix-B challenge dose
- subgroup 3: anti-HBs concentration < 10 mIU/mL at baseline and anti-HBs concentration < 10 mIU/mL after the Engerix-B challenge dose

10. Ethical issues

This study will be submitted for formal review and approval to the Ethics Committee of the University Hospital Antwerp. No participants will be enrolled, nor will any study specific and subject related activities be performed before written approval from this body is obtained.

In this observational study all participants will be adults who received complete vaccination with Infanrix Hexa during infancy. There will be a close collaboration with the external occupational health service at the University of Antwerp (Mensura) for recruitment and blood draws at several visits. In addition, anti-HBs antibody results of day 1 and day 29 will be shared between the study team and the team members of Mensura involved in this study to make sure that the participants are offered a challenge vaccination or complete re-vaccination against hepatitis B (if applicable) at Mensura in order for them to be protected before starting their internship. In this way, the study will not intervene with or jeopardize the normal preventive procedures foreseen by Mensura to protect these students before the start or their traineeship.

In the ICF this exchange of anti-HBs antibody results with Mensura will be explained to the subjects and their permission will be asked.

Risk to the participants is considered to be minimal, as minimally invasive (blood sample) procedures will be undertaken.

The study will be carried out according to the principles stated in the World Medical Association's Declaration of Helsinki (Ethical Principles for Medical Research Involving Human Subjects) as amended in 2013, in accordance with all applicable regulations and established

international scientific standards. The University of Antwerp has concluded a no-fault insurance agreement to cover any harm or injury to research participants which is caused directly or indirectly by their participation in the research study.

11. Finance and resource use

This non-commercial study is financed By GSK Biologicals.

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13. Contact information

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Appendix 1: definition of adverse event and serious adverse events

1. Definition of an adverse event

An adverse event (AE) is any untoward medical occurrence in a clinical study participant, temporally associated with the use of a study intervention, whether or not considered related to the study intervention.

Note: an AE can therefore be an unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease (new or exacerbated) temporally associated with the use of a study intervention.

Events meeting the AE definition

15. Any abnormal laboratory test results (hematology, clinical chemistry, or urinalysis) or other safety assessments (e.g., ECG, radiological scans, vital signs measurements), including those that worsen from baseline, considered clinically significant in the medical and scientific judgement of the investigator (i.e., not related to progression of underlying disease).
16. Exacerbation of a chronic or intermittent pre-existing condition including either an increase in frequency and/or intensity of the condition.
17. New condition detected or diagnosed after study intervention administration even though it may have been present before the start of the study.
18. Signs, symptoms, or the clinical sequelae of a suspected intervention – intervention interaction.
19. Signs,symptoms, or the clinicalsequelae of a suspected overdose of eitherstudy intervention or a concomitant medication. Overdose per se will not be reported as an AE/SAE unless it is an intentional overdose taken with possible suicidal/self-harming intent. Such overdoses should be reported regardless of sequelae.
20. Events that occur as a result of protocol-mandated procedures (i.e. invasive procedures, modification of participant's previous therapeutic regimen).

Events not meeting the AE definition

21. Any clinically significant abnormal laboratory findings or other abnormal safety assessments that are associated with the underlying disease, unless judged by the investigator to be more severe than expected for the participant's condition.
22. Medical or surgical procedure (e.g., endoscopy, appendectomy): the condition that leads to the procedure is the AE.
23. Situations in which an untoward medical occurence did not occur (social and/or convenience admission to a hospital, admission for routine examination).
24. Anticipated day-to-day fluctuations or pre-existing disease(s) or condition(s) present or detected at the start of the study that do not worsen. Pre-existing diseases will be recorded in the medical history section of the eCRF.
25. Hospitalization for elective treatment of a pre-existing condition (known or diagnosed before signing the informed consent) that did not worsen from baseline.

2. Definition of a serious adverse event

A serious adverse event (SAE) is defined as any untoward medical occurrence that, at any time, meets one or more of the criteria listed:

26. Results in death
27. Is life threatening
    - The term ‘life-threatening’ in the definition of ‘serious’ refers to an event in which the participant was at risk of death at the time of the event. It does not refer to an event, which hypothetically might have caused death, if it were more severe.

28. Requires inpatient hospitalization or prolongation of existing hospitalization
    - In general, hospitalization signifies that the participant has been admitted (usually involving at least an overnightstay) at the hospital or emergency ward for observation and/or treatment that would not have been appropriate in the physician's office or outpatient setting. Complications that occur during hospitalization are AE. If a complication prolongs hospitalization or fulfills any other serious criteria, the event is serious. When in doubt as to whether 'hospitalization' occurred or was necessary, the AE should be considered serious.
    - Hospitalization for elective treatment of a pre-existing condition that did not worsen from baseline is not considered an AE.
29. Results in persistent or significant disability/incapacity
    - The term disability means a substantial disruption of a person's ability to conduct normal life functions.
    - This definition is not intended to include experiences of relatively minor medical significance such as uncomplicated headache, nausea, vomiting, diarrhea, influenza, and accidental trauma (e.g., sprained ankle) that may interfere with or prevent everyday life functions but do not constitute a substantial disruption.
30. Is a congenital anomaly/birth defect in the offspring of a study participant
31. Other situations:
    - Medical or scientific judgment should be exercised by the investigator in deciding whether SAE reporting is appropriate in other situations such as significant medical events that may jeopardize the participant or may require medical or surgical intervention to prevent one of the other outcomes listed in the above definition. These events should be considered serious. Examples of such events include invasive or malignant cancers, intensive treatment for allergic bronchospasm, blood dyscrasias, convulsions, or development of intervention dependency or intervention abuse.

Appendix 2: Assessment of adverse events

1. Assessment of intensity

The investigator will make an assessment of intensity for each SAE reported during the study and assign it to one of the following categories:

- **Mild**: A type of AE that is usually transient and may require only minimal treatment or therapeutic intervention. The event does not generally interfere with usual activities or daily living.
- **Moderate**: A type of AE that is usually alleviated with additional specific therapeutic intervention. The event interferes with usual activities of daily living, causing discomfort but poses no significant or permanent risk of harm to the research participant.
- **Severe**: A type of AE that interrupts usual activities of daily living, orsignificantly affects clinical status, or may require intensive therapeutic intervention.

The investigator will assess the maximum intensity that occurred over the duration of the event for all SAEs recorded during the study. The assessment will be based on the investigator's clinical judgment.

2. Assessment of causality

The investigator will assess the relationship between study intervention and each occurrence of each SAE. The investigator will use clinical judgment to determine the relationship.

A reasonable possibility of a relationship conveys that there are facts, evidence, and/or arguments to suggest a causal relationship, rather than a relationship cannot be ruled out.

Alternative causes, such as underlying disease(s), concomitant therapy, and, and other risk factors, as well as the temporal relationship of the event to study intervention administration, will be considered and investigated.

The final assessment by the investigator will be binary of nature. That is, SAEs for which the causality is assessed as likely, very likely, definitely, suspected (reasonable possibility), cannot be ruled out, unlikely, or probably are considered related. In contrast, SAEs for which the causality is assessed as not related, dubiously, or remotly are considered not related.

3. Assessment of outcomes

The outcome of all SAEs will be recorded during the study as:

- Recovered/resolved
- Recovering/resolving
- Not recovered/not resolved
- Recovered with sequelae/resolved with sequelae
- Fatal

Signature page - Sponsor

Study Title	The persistence of vaccine-induced HBsAg-specific antibodies and immune memory in adults after complete primary vaccination with the GlaxoSmithKline (GSK) hexavalent Infanrix vaccine at infancy
Version, date	1, 26JUN2026

'This clinical study protocol has been reviewed and approved by the Sponsor in order to ensure compliance with Good Clinical Practice.'

Sponsor Representative Signature Date	

Signature page - Investigator

Study Title	The persistence of vaccine-induced HBsAg-specific antibodies and immune memory in adults after complete primary vaccination with the GlaxoSmithKline (GSK) hexavalent Infanrix vaccine at infancy
Version, date	1, 26JUN2026

'I have read this clinical study protocol and agree that it contains all information necessary for proper conduct of the study. I will carry out the study as outlined herein and will complete the study within the designated time.'

Principal Investigator Signature Date	
Co-Investigator Signature Date	