1.1 Title

Phase I clinical trial to evaluate the safety and immunogenicity of StreptInCor, a synthetic vaccine against Streptococcus pyogenes, in healthy adult volunteers

1.2 Protocol Summary

This is a Phase I/IIa, randomized, double-blind, placebo-controlled, dose-escalation clinical trial to test the candidate vaccine StreptInCor. The study will include four different doses (25 µg, 50 µg, 100 µg, and 200 µg) of StreptInCor produced under Good Manufacturing Practices (GMP) and formulated with aluminum hydroxide as the vaccine adjuvant. The adjuvant alone will be used as a placebo in this trial. Five groups, each consisting of twelve healthy adult volunteers, will receive randomly assigned two doses of vaccine or placebo with a 28-day interval, plus a booster dose 6 months after the initial vaccination. During the recruitment process, volunteers aged 18-45 years will undergo general health evaluation and serological testing to confirm the absence of HIV and autoimmune diseases. Second-degree relatives or individuals with a history of Acute Rheumatic Fever (ARF) or Rheumatic Heart Disease (RHD), as well as those with chronic or recurrent infections linked to S. pyogenes, will be excluded. All volunteers must sign an informed consent form before any procedures.

Initially, volunteers assigned to receive the lowest dose (25 µg) and three volunteers from the placebo group will start the vaccination schedule to maintain blinding. One month after the second vaccination, all volunteers will undergo a safety evaluation, and the results will be submitted to the Data Safety Monitoring Committee (DSMC). Only upon favorable review by the DSMC will the booster dose be administered. After the DSMC reviews the safety of the three doses, the second phase of the study can begin. In this phase, volunteers selected for the intermediate-low dose (50 µg) and three additional placebo volunteers will start the vaccination schedule. Similar to the previous phase, the DSMC will assess safety data one month after the second vaccination. If the review is favorable, a six-month booster will be given and the safety evaluated again. If approved, the third phase proceeds, involving the intermediate-high dose (100 µg) and another three placebo volunteers. The DSMC will review safety after the second vaccination before authorizing the booster. Upon approval, the third dose will be administered, followed by another safety review by the DSMC before starting the fourth phase. This final phase will involve volunteers receiving the high dose (200 µg) and three placebo controls. Similar safety assessments will occur, and if favorable, a six-month booster will be administered and reviewed.

A total of 60 volunteers will participate, with 12 per group and 15 per phase. Safety (toxicity) will be monitored by the DSMC after each booster in all phases. The DSMC operates based on the Operational Guidelines for the Establishment and Operation of Data and Safety Monitoring Committees by the Ministry of Health (2008). Safety parameters will include serological markers for

autoimmune diseases. Safety follow-up will last up to one year after the initial vaccination. The study or any of its phases may be suspended at any time if a moderate to severe adverse event possibly related to the vaccine occurs. The DSMC will then evaluate whether to halt or restart the step or the entire study.

The primary efficacy endpoint will be at least a fourfold increase in IgG antibody levels after the last dose compared to pre-vaccination levels. All samples from the same volunteer will be processed simultaneously to avoid bias, and analyses will be performed blindly by a team without access to patient data. Additional efficacy parameters include detection of other antibody classes, assessment of antibody functionality (surface binding, inhibition of bacterial invasion/adhesion, and phagocytosis induction), cellular immune responses such as cytokine profiling, antigen-specific cell proliferation, and induction of memory T cells. The dose with the highest seroconversion rate and an acceptable safety profile will be selected for Phase II trials.

Keywords: Acute rheumatic fever (ARF), Rheumatic heart disease (RHD), Streptococcus pyogenes, Group A streptococcus (GAS), Vaccine development program**.**

1.3 Literature Review / Current State of Knowledge on the Project Topic

Streptococcus pyogenes causes various severe infections: Acute Rheumatic Fever (ARF) and Rheumatic Heart Disease (RHD), acute glomerulonephritis, uncomplicated pharyngitis, and impetigo. The prevalence of RHD has been increasing annually worldwide and is estimated to continue this pattern for the next 20 to 29 years. Although it affects different regions, the most impoverished populations face the highest incidence and early mortality from the disease. In 2019, there were over 40 million cases globally, with 306,000 deaths (Roth et al., 2020). Projections forecast that the prevalence of heart failure due to RHD will reach nearly 27 per 100,000 inhabitants, totaling almost 3 million cases by 2030, with the highest prevalences in developing regions (Hu et al., 2002).

The prevalence of ARF and RHD within a community reflects the level of primary preventive care and is also associated with social factors, demonstrating failures in early detection and intervention by health systems, which lead to more severe disease forms (Faleiro et al., 2023).

In Brazil, approximately 30,000 cases of ARF occur annually, with an incidence of 7 cases per thousand school-aged children (Faleiro et al., 2023). The mortality rate from RHD increased by over 40% between 1998 and 2016, though these numbers are likely underestimated due to lack of surveillance. Hospitalization costs for RHD increased by 264% during the same period, exerting a

significant impact on the Unified Health System (SUS), especially given that RHD is a preventable disease (Figueiredo et al., 2019). Data from 1996 indicate that ARF sequelae are among the leading causes of cardiac surgery in Brazil (Snitcowsky, 1996). More recent data estimate that Brazil performs 10.4 cardiac surgeries per million inhabitants, and RHD is among the cardiovascular diseases requiring this intervention most frequently (Faleiro et al., 2023). Additionally, most of these surgeries are not definitive, as many patients receive bioprostheses and lack knowledge or access to the oral anticoagulation required for metallic prostheses (Tarasoutchi et al., 2003).

Unfortunately, none of this data can truly reflect the actual impact of ARF, as the disease is generally asymptomatic (Tubridy-Clark and Carapetis, 2007), and only about 3% of patients require hospitalization. When analyzing the number of hospital admissions due to ARF, it is important to consider that for each diagnosed case, there are many asymptomatic or undiagnosed patients who seek medical care decades after the initial ARF episode, often when severe heart failure has already developed due to significant valvular lesions and requires surgical correction. Data on cardiac surgeries related to ARF confirm that the disease has remained active in recent years (Tarasoutchi et al., 2003).

Attempts to develop a vaccine against ARF began over 100 years ago, with the last 80 years focusing mainly on the M protein of S. pyogenes. Currently, there are four vaccines based on the M protein. In addition to the StreptInCor vaccine discussed in this document, StreptAnova is a multivalent vaccine based on the N-terminal portion of the protein, which is a highly variable region and has already been tested in a phase I clinical trial with promising results. The J8/S2 Combivax and P*17/S2 Combivax vaccines are based on peptides from the conserved C-terminal region of the M protein, combined with non-M proteins, and have entered phase Ia clinical trials.

Besides these, vaccines that are not based on the M protein are being developed focusing on other antigens with broad coverage among S. pyogenes types. These include non-protein antigens such as the Group A Carbohydrate (GAC) and protein antigens like Streptolysin O toxin, SpyCEP protease, SpyAD adhesin, C5a peptidase enzyme, pilus, and others, which may be combined or used separately (Walkinshaw et al., 2023).

In our work, we studied an amino acid sequence present at the C-terminal position of the M protein to develop a subunit vaccine capable of inducing protection against different S. pyogenes strains. To define the vaccine epitope, we tested a large panel of approximately 900 sera and peripheral blood mononuclear cell (PBMC) samples, which allowed us to identify immunodominant B and T epitopes and to construct a vaccine candidate comprising 55 amino acid residues from this sequence (Guilherme et al., 2006;

Guilherme et al., 2009). We demonstrated that this vaccine epitope, called StreptInCor (medically identified), has structural features in three dimensions that make it recognizable by any human leukocyte antigen (HLA) class II, resulting in activation and differentiation of effector and memory T cells, as well as inducing B lymphocytes to produce specific antibodies (Guilherme et al., 2011).

1.4 Justification for the Study

ARF is one of the main causes of acquired heart disease in developing countries, and preventive antibiotic therapy is not effectively used in populations with limited access to healthcare. Therefore, a vaccine for this condition is highly desirable, as immunization would provide these populations with protection against the late lethal effects of streptococcal infection.

1.5 Potential/Intended Use of Study Results

Vaccination against ARF could be the most effective way to reduce the disease incidence in populations with limited access to healthcare. Since rheumatic sequelae involve significant costs to the healthcare system, vaccination may also represent the best cost-benefit approach to controlling ARF.

2. Objectives and Proposal

2.1 Primary Objective

The clinical development aims to evaluate the ability of the synthetic vaccine against *Streptococcus pyogenes* to induce the production of protective antibodies (immunogenicity) and to assess whether these antibodies do not trigger autoimmune or deleterious reactions (safety). The vaccine will be administered in different doses (25 µg, 50 µg, 100 µg, and 200 µg) and compared with placebo in healthy adult volunteers.

In the first phase of the trial, volunteers assigned to receive the lowest dose (25 µg) and three volunteers from the placebo group, to maintain the study's blind, will begin the vaccination schedule. One month after the second dose, all volunteers will be re-evaluated for safety, and the results will be submitted to the Data and Safety Monitoring Committee (DSMC). Only after a favorable opinion from the DSMC will the booster dose be administered to the group.

Following the DSMC's assessment of the safety of the three doses, the second phase of the study may start. In this phase, volunteers selected to receive the intermediate-low dose (50 µg) and three additional placebo volunteers will begin the vaccination schedule. Similar to the previous phase, the DSMC will analyze safety data one month after the second vaccination. If the DSMC's opinion is favorable, the group will receive a six-month booster and will be evaluated again by the DSMC.

If the DSMC’s assessment remains favorable, the third phase will commence. This will include the high-intermediate dose group (100 µg) and three more placebo volunteers. The DSMC will review safety data after the second vaccination before approving or not the booster dose. If approved, the third dose will be administered, followed by another safety assessment by the DSMC to authorize the start of the fourth phase.

For the fourth phase, volunteers assigned to receive the high dose (200 µg) and three additional placebo volunteers will begin the vaccination schedule. As in the previous step, the DSMC will analyze safety results one month after the second vaccination. If the results are favorable, this group will receive a booster in six months, with subsequent reassessment by the DSMC.

2.2 Secondary Objectives

a) To determine the rate of reactions following immunization, i.e., reactogenicity, signs of ARF, symptoms, and other events, with the four different doses of StreptInCor and placebo up to 6 months after the administration of the last dose;

b) To determine the incidence of cross-reactive antibody cases against cardiac myosin peptides after immunization with the four different doses of StreptInCor or placebo, up to six months after the last dose;

c) To determine the levels of specific antibodies against StreptInCor (IgG, IgA, and IgM) after immunization with the four different doses of StreptInCor or placebo, up to six months after the last dose;

d) To assess the functionality of the antibodies generated after immunization with the four different doses of StreptInCor or placebo, up to six months after the last dose;

e) To evaluate the specific cellular immune response against StreptInCor after immunization with the four doses of StreptInCor or placebo, up to six months after the last dose;

f) To assess immunological markers in cells and cytokine patterns after immunization with the four doses of StreptInCor or placebo, up to six months after the last dose;

g) To identify HLA class II alleles in individuals immunized with the four doses of StreptInCor or placebo;

h) To correlate HLA class II types with the incidence of adverse events after immunization with the four doses of StreptInCor or placebo, up to six months after the last dose;

i) To correlate HLA class II types with the immune response after immunization with the four doses of StreptInCor or placebo, up to six months after the last dose.

2.3 Hypotheses or Questions

a) The administration of StreptInCor at doses of 25 µg, 50 µg, 100 µg, and 200 µg has an overall incidence rate of adverse events post-immunization of less than 10%;

b) The administration of StreptInCor at doses of 25 µg, 50 µg, 100 µg, and 200 µg is not associated with the development of antibodies with cross-reactivity against human tissues;

c) The administration of StreptInCor at doses of 25 µg, 50 µg, 100 µg, and 200 µg triggers an immune response characterized by a fourfold increase in anti-StreptInCor antibodies, capable of inhibiting mucosal adhesion and inducing phagocytosis of S. pyogenes;

d) There is no relationship between HLA class II antigens and a higher incidence of adverse events, nor with a different immune response after administration of StreptInCor.

3. Study Design

3.1 General Approach and Study Design

This is a randomized, double-blind, placebo-controlled Phase I/IIa clinical trial with dose escalation to test four different doses of the candidate vaccine StreptInCor.

3.2 Primary Outcomes

• Safety outcome: The primary safety objective will be the absence of serious adverse events following immunization that have a reasonable causal relationship with the studied product in the StreptInCor and placebo groups.

• Immunogenicity outcome: The primary immunogenicity outcome will be at least a fourfold increase in IgG antibody levels against the StreptInCor peptide, 6 months after the last immunization.

3.3 Exploratory Outcomes

• Incidence of cross-reactive antibodies against cardiac myosin peptides up to six months after the last vaccination;

• Variation in anti-StreptInCor IgA levels from baseline to 6 weeks after the last immunization;

• Variation in anti-StreptInCor IgM levels from baseline to 6 weeks after the last immunization;

• Variation in inhibition of S. pyogenes mucosal adhesion from baseline to 6 weeks after the last immunization;

• Variation in induction of S. pyogenes phagocytosis from baseline to 6 weeks after the last immunization;

• Variation in cytokine detection from baseline to 6 weeks after the last immunization;

• Variation in cellular markers from baseline to 6 weeks after the last immunization;

• Correlation of HLA class II antigens with adverse reactions and immunogenicity of StreptInCor.

3.4 Randomization

Participants will be divided into two groups for each stage: either placebo or the active study product. An external person will generate the randomization list, which will be sent to the production area to ensure the correct labeling of study products. An independent electronic randomization system will be used, which operates separately and is not linked to any third-party system. Its validation is conducted prior to each new clinical study, guided by a risk-based approach. The process is described in Operational Procedures (POPs), notably the "VSC 001 - Validation of Computerized Systems," which is maintained within a Quality System. Evidence of the validation execution is collected during the process. At the end of each validation, a “Final Validation Report” is prepared.

Products will be sequentially coded and distributed according to the order of enrollment. The investigation site will have a distribution list corresponding to the study products.

The ratio of study product to placebo for each dose will be 4:1. Since four doses are tested, the final study will aim for an equal number of participants for each dose group and the placebo group.

3.5 Confidentiality

The study products (active and placebo) will be manufactured in accordance with Good Manufacturing Practices (GMP). Both the active products and placebos will be similar in organoleptic characteristics and presentation to prevent accidental identification by the study staff or participants. A manufacturing area independent of the investigation sites will be responsible for labeling the products according to the randomization list. Labels will identify products only by a sequential code. This labeling process will also adhere to GMP principles.

Researchers, participants, and laboratory personnel responsible for analyses will be blinded to the distribution of study groups.

Sealed envelopes containing the individual secret code will be sent to the study sites and can be opened only upon justified request from the medical provider responsible for the participant’s care. Since the studied product has no specific antidote, opening these envelopes generally does not provide useful information for medical management.

The secret code will only be revealed once the database with clinical and laboratory information is complete and ready for analysis.

4. **Population Study**

4.1 Description, Population Source, and Area Scope

Participants will be selected from the general adult population residing in São Paulo, at study sites, companies, educational centers, and areas with high foot traffic such as metro stations, shopping malls, and avenues. Recruitment material (posts on social media, posters, online advertisements, etc.) will be released only after written approval by the respective ethics committees.

4.2 Case Definitions

• Healthy volunteer: An individual with no confirmed diagnosis of disease or infection that impairs immune response;

• Person with prior rheumatic fever: An individual with a presumptive or confirmed diagnosis of ARF. A history of compatible symptoms, without other explanation, is also considered a presumptive diagnosis;

• Family history of rheumatic fever: Defined as involving first- or second-degree blood relatives among its members.

4.3 Inclusion Criteria

• Healthy volunteers of either sex, aged between 18 and 45 years;

• Availability for all procedures throughout the study period;

• Providing free and informed consent to participate in the study.

4.4 Exclusion Criteria

• Participation in clinical trials within the last year;

• Participation in cohort studies;

• Diagnosis of concomitant infections or diseases that may affect immunity, including active HIV infection, hepatitis B, hepatitis C, diabetes mellitus, neoplasms, or autoimmune diseases;

• Current or previous diagnosis, or family history, of ARF, chorea, obsessive-compulsive disorder, or glomerulonephritis;

• Current or previous diagnosis of heart diseases;

• Severe asthma or chronic obstructive pulmonary disease (COPD);

• Abnormal neurological assessment, especially chorea;

• Use of treatments that may affect immunity in the last four weeks, including immunomodulators, corticosteroids (used systemically for two weeks or more), or antineoplastic agents;

• Use of treatments that may affect heart valves in the last four weeks or planned during the study, including fenfluramine and dexfenfluramine;

• Renal insufficiency, defined as estimated creatinine clearance below 45 ml/min/1.73m^2;

• History of intolerance or allergy to any component of the study product, including antigen or adjuvant;

• Presence of valvular abnormalities or structural changes in the heart identified by echocardiography;

• Abnormal electrocardiogram;

• Evidence or suspicion of recent S. pyogenes infection based on clinical symptoms within the last four weeks;

• Pregnancy, breastfeeding, or intention to become pregnant during the study period (only for female participants);

• Any other condition that, in the opinion of the investigators, could interfere with the study process, including sample size and statistical power.

This is an exploratory Phase I study. No formal sample size calculation has been performed. Twelve participants are expected per dose group and the placebo group. In the first stage, 15 participants are planned: 12 will receive the low dose and three will receive placebo; in the next step, there will be 12 with the intermediate-low dose and three placebo; the following step will include 12 with the intermediate-high dose and three placebo; finally, 12 will receive the high dose and the remaining three will be in the placebo group.

4.5 **Screening and Inclusion**

Potential study participants will undergo a screening consultation, during which, after providing informed consent, their medical history, demographic data, and, if available, hospital records will be reviewed by a physician to assess their eligibility for inclusion in the study.

At the screening visit, all potential participants not excluded for other reasons will have blood samples collected and an echocardiogram scheduled. Participants who become ineligible due to laboratory results may undergo additional testing up to two weeks after the initial blood collection.

Once a dose group is complete, subsequent potential participants may have their echocardiogram and sample collection delayed until the next dose group’s enrollment is authorized.

Participant enrollment can occur at any time within 8 weeks following the screening examinations. The physician must verify whether any abnormal results are related to a transient condition. If so, a repeat test can be scheduled during the same 8-week screening period. If the repeat test results fall within acceptable parameters, the volunteer may be enrolled in the study. If the patient does not enroll within this 8-week window, the screening process must be restarted, and a new consent obtained. A patient may be evaluated only twice for this study.

All patients must have blood samples collected to obtain an initial assessment of the following parameters:

Complete blood count with platelets;

C-reactive protein;

ASLO (Anti-streptolysin O);

Anti-DNAse B, Troponin;

CKMB (Creatine phosphokinase MB fraction);

Liver function tests: AST (Aspartate Transaminase), ALT (Alanine Transaminase), Bilirubin;

Renal function: Urea, Creatinine;

Amylase;

ANF (Antinuclear Factor);

Anti-HIV;

HBsAg and Anti-HBs;

Anti-HCV;

Rheumatoid factor;

Complement;

Immunosurveillance panel (to check for the presence of cross-reactive antibodies against cardiac myosin peptides);

Tryptase;

HLA class II panel (HLA-DRB1, DRB3, DRB4, DRB5, and DQB1);

Immunogenicity panel (to establish baseline titers of specific anti-StreptInCor antibodies and their capacity to neutralize S. pyogenes infection in vitro via inhibition of bacterial adhesion/invasion in Hep cells).

Additionally, Urinalysis, electrocardiogram, and echocardiogram will be performed during screening. Pregnancy tests will be conducted on all women of childbearing age.

Screening must be completed within 28 days prior to randomization. If results are abnormal, a new set of tests may be repeated.

5. Study Procedures

5.1 Study Product

The candidate vaccine product, StreptInCor, is composed of 55 amino acid residues (Guilherme et al., 2009) (INPI patents 0501290/0604997-4, PCT-BR07/000184) and is produced as a synthetic peptide under Good Manufacturing Practices (GMP). It is structurally stable at low and high temperatures (4°C to 90°C), exhibiting biological and chemical properties that make it a universal vaccine (Massel, 1997).

5.2 Allowed and Prohibited Concomitant Medications

During the study period, participants should avoid any medication without medical prescription. Medications for mild illnesses and prescribed medications that do not interfere with immunity are permitted. The use of topical corticosteroids such as creams, nasal sprays, or inhalers is also allowed. Any medication used during the study must be recorded in the patient's medical record.

Use of any medication that could interfere with immunity—such as immunomodulators, systemic corticosteroids (used for two weeks or more), or antineoplastic agents—is not permitted and may lead to participant withdrawal from the study. Participants must inform the study team as soon as possible if they receive prescriptions for any of these medications. In such cases, the study team

will advise the participant to discontinue the study product until a consultation is scheduled to assess ongoing participation. During this consultation, the physician may decide to discontinue the participant if they determine that the medication use could interfere with adherence to study procedures.

The use of alcohol or sporadic use of illegal or prescribed drugs is not, by itself, a reason to withdraw a participant from the study. However, a member of the study team may decide to withdraw the participant if they consider that such use could interfere with adherence to the study procedures.

5.3 Procedures for Monitoring and Ensuring Adherence to the Study Product

Participants will receive the study product only at the location where the study is conducted. The study product will be stored at the study site following the manufacturer's recommendations. The site must keep records of each administration of the product and update the stock accordingly.

5.4 Immunogenicity Assessment

5.4.1 Immunogenicity Parameters

The primary efficacy parameter will be a fourfold increase in IgG antibody levels against StreptInCor, six months after the last immunization. This parameter is based on a study of a vaccine containing four recombinant proteins adsorbed onto aluminum hydroxide and the M protein, where the geometric mean titers of IgG antibodies against the peptides were used, with a fourfold rise to assess participant immunogenicity (McNeil et al., 2005; McNeil et al., 2006).

Secondary parameters will include increases in IgA and IgM antibodies against StreptInCor, inhibition of adhesion to mucosa, induction of phagocytosis of S. pyogenes, as well as changes in cytokine levels and cellular markers up to six months post-final vaccination.

The primary efficacy evaluations for this exploratory study will be based on the per-protocol analysis, meaning only participants who comply with study procedures will be included. Intention-to-treat analysis will be considered a secondary efficacy analysis.

5.4.2 Methods for Evaluating Efficacy Parameters

The analytical methods described here will be performed by a research laboratory and will be validated in later phases of the clinical development of the product.

5.4.2.1 S. pyogenes Collection

A collection of S. pyogenes isolates is maintained, recovered from samples of the oropharynx, tonsils, skin, bloodstream infections, or other sources from patients attended at the Hospital das Clínicas of the Faculty of Medicine, University of São Paulo (HCFMUSP), Brazil. These bacteria were identified based on characteristic hemolysis on blood agar, sensitivity to bacitracin, emm gene amplification, and sequencing using the BLAST 2 database (National Center for Biotechnology Information - http://www.ncbi.nlm.nih.gov/BLAST) and the CDC (Center for Disease Control and Prevention - Department of Health and Human Services - http://www.cdc.gov). These isolates will be used in assays such as Surface Antibody Binding, Streptococcus Invasion/Adhesion, and Opsonophagocytosis assays.

5.4.2.2 Antibody Detection

96-well high-binding plates will be precoated with StreptInCor peptide in carbonate buffer for 1 hour at 37°C. After washing with PBS-Tween 20 and blocking with PBS-Bovine Serum Albumin (BSA) for 1 hour at 37°C, the serum samples from study participants

will be added in PBS-BSA buffer and incubated overnight at 4°C. After washing, plates will be incubated with human anti-IgG, anti-IgA, or anti-IgM antibodies conjugated to peroxidase for 1 hour at 37°C. Following another wash, the substrate o-phenylenediamine (OPD) will be added for 30 minutes at 37°C, and the reaction stopped with H_2SO_4. Absorbance will be measured in an automated plate reader at 490 nm.

5.4.2.3 Antibody Binding to Bacterial Surface

S. pyogenes isolates will be cultured in THY (Todd-Hewitt) medium at 37°C until reaching OD_{600}nm = 0.4, washed with PBS-BSA, and then incubated with the serum from study participants for 30 minutes. After washing again, the bacteria will be incubated with human anti-IgG antibodies conjugated to a fluorophore for 30 minutes. Following washing, the samples will be fixed with 1% paraformaldehyde, and 10,000 events will be acquired using flow cytometry.

***5.4.2.4 Invasion/Adhesion of Streptococcus**

Semi-confluent monolayers of HEp2 cells (human laryngeal carcinoma) will be cultured in modified Eagle's Medium (D-MEM) supplemented with 1 mM L-glutamine and 10% heat-inactivated fetal bovine serum (FBS) at 37°C and 5% CO_2. Serum samples from participants will be incubated with suspensions of *S. pyogenes* (7×10^5 bacteria) for 1 hour at 37°C. Then, HEp2 cells will be infected for 2 hours at 37°C. The cells will be washed once with PBS, treated with trypsin, and lysed. The cell lysates will be diluted in PBS and plated on blood agar to quantify viable bacteria, expressed as colony-forming units (CFU). The percentage of inhibition will be calculated based on the CFUs in conditions incubated with serum (from vaccinated and placebo groups) versus without serum.

5.4.2.5 Cell Proliferation

Proliferation assays will be performed using the CellTrace™ CFSE Cell Proliferation Kit (Invitrogen). Peripheral blood mononuclear cells (PBMCs) will be isolated from study participants via density gradient separation. Cells will be washed with PBS, labeled with carboxyfluorescein succinimidyl ester (CFSE), and incubated at 37°C for 10 minutes. The labeling process will be blocked by adding cold RPMI medium with 10% FBS and incubating at 4°C for 5 minutes. Cells will then be washed again, resuspended in RPMI with 10% FBS, and stimulated with StreptInCor peptide for 5 days at 37°C and 5% CO_2. After incubation, cells will be washed and analyzed by flow cytometry to assess cell proliferation.

5.4.2.6 Cytokine Detection

The production of pro-inflammatory cytokines (IFN-γ and TNF-α) and anti-inflammatory cytokines (IL-4 and IL-10) at baseline in serum and after PBMC stimulation will be detected using Cytometric Bead Array (CBA). PBMCs will be obtained via density gradient separation. Cells will be resuspended in RPMI medium containing 10% FBS and stimulated with StreptInCor peptide for 5 days at 37°C and 5% CO_2. For cytokine quantification, 35 µL of capture beads coated with specific antibodies and fluorescence, 50 µL of stimulated cells or serum, and 25 µL of detection reagent will be mixed and incubated for 2 hours at room temperature in the dark. Samples will then be washed and resuspended for flow cytometry analysis. Cytokines will be quantified in pg/mL based on a standard curve with known concentrations.

5.4.2.7 Opsonophagocytosis

The opsonophagocytosis assay will use HL-60 cells (a human promyelocytic leukemia cell line) cultured in RPMI medium containing 10% FBS and differentiated into neutrophil-like cells by adding 0.9% dimethylformamide (DMF) to the culture medium for 5 days. The cells will be washed and resuspended to a concentration of 2×10^7 cells/mL.

S. pyogenes isolates will be washed and suspended at a concentration of 10^5 CFU/mL; 10 µL of this suspension will be added to a 96-well plate prepared for cell culture, along with serial dilutions of serum from study participants. The plates will be incubated for 30 minutes at room temperature. Subsequently, 20 µL of HL-60 cells (at a phagocyte:bacteria ratio of 400:1) and a source of complement will be added. The plates will then be incubated for 45 minutes at 37°C with 5% CO_2 and constant agitation.

To stop the reaction, samples will be incubated on ice for 20 minutes. After incubation, 5 µL of each sample will be plated onto solid THY medium and incubated at 37°C for about 15 hours. Phagocytosis will be considered significant when the number of non-phagocytosed bacteria, determined by colony count, shows at least a 50% reduction compared to controls without immune serum.

5.4.2.8 Cell Markers

The analysis of cellular markers aims to identify potential biomarkers relevant to the protection generated by the vaccine. Various cellular markers will be used to identify memory T cells in PBMCs (peripheral blood mononuclear cells) from study participants by flow cytometry. For example: naïve T cells (TN), central memory T cells (TCM), effector memory T cells (TEM), tissue-resident memory T cells (TRM), as well as the phenotype of regulatory T cells and cytokine profiles of Th1 and Th2 cells.

5.4.2.9 HLA Typing

Participants' DNA will be extracted from whole blood and amplified using polymerase chain reaction (PCR). They will then be typed for HLA-DRB1, DRB3, DRB4, DRB5, and DQB1 alleles using the commercial LABType™ kit (One Lambda, CA, USA), following the manufacturer’s instructions.

5.5 Safety Assessment

5.5.1 Safety Parameters

The primary safety parameter will be the rate and severity of adverse events reasonably related to the study product. All individuals who receive at least one dose of the study product will be included in the safety analysis.

5.5.2 Methods for Assessing Safety Parameters

5.5.2.1 Overall Safety Evaluation

Beyond the general safety assessment, safety monitoring will focus on detecting symptoms induced by the vaccine that could suggest a history of Rheumatic Fever (RF). All participants will be monitored at 2, 4, 8, 18, and 26 weeks post-vaccination.

Adverse events will be evaluated using clinical and laboratory parameters. All adverse events will be classified by intensity and their causal relationship with the study product will be assessed.

Routine safety evaluations will include parameters described in Tables 1 and 2. Participants will be observed for two hours after vaccination to monitor immediate reactions (see Tables 1 and 2). A medical professional specializing in adverse drug reactions (clinical allergist) will be present to closely monitor and manage any immediate hypersensitivity reactions during this period.

If any participant experiences an immediate hypersensitivity reaction, serum tryptase levels will be collected within two hours of symptom onset. Some reactions may occur immediately, while others may develop later; therefore, participants will complete questionnaires to report local and systemic adverse events between visits.

All adverse events will be evaluated and classified according to the criteria detailed in Tables 3 to 8.

Table 1. Severity of Local Reactions to the Investigational Product
Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)

Local Reaction to the Injectable Product	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Pain (including tenderness)	Does not interfere with activity	Repeated use of non-opioid analgesics >24 hours or interferes with activity	Use of any analgesic or prevents daily activity	Visit to ER or hospitalization
Erythema (redness)*	2.5 – 5 cm	5.1 – 10 cm	> 10 cm	Necrosis or sloughing dermatitis
Induration (hardening)**	2.5 – 5 cm, no interference with activity	5.1 – 10 cm or interferes with activity	>10 cm or prevents daily activity	Necrosis
Swelling (edema)**	2.5 – 5 cm, no interference	5.1 – 10 cm or interferes	>10 cm or prevents daily activity	Necrosis

In addition to classifying the greatest diameter of the local reaction, measurement should be recorded as a continuous variable.

Endurance/inflammation should be assessed and graded using a functional scale and actual measurement.

In case of an acute allergic reaction, the patient should be properly treated at the emergency room. If necessary, hospitalization will be arranged.

Table 2. Severity of Vital Signs Related to Adverse Effects
Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)

Vital Signs *	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Fever (°C) **	38.0 – 38.4	38.5 – 38.9	39.0 – 40	> 40
Tachycardia - beats per minute	101 – 115	116 – 130	> 130	ER visit or hospitalization due to arrhythmia
Bradycardia - beats per minute ***	50 – 54	45 – 49	< 45	ER visit or hospitalization due to arrhythmia
Systolic hypertension - mm Hg	141 – 150	151 – 155	> 155	ER visit or hospitalization due to malignant hypertension
Diastolic hypertension - mm Hg	91 – 95	96 – 100	> 100	ER visit or hospitalization due to malignant hypertension
Systolic hypotension - mm Hg	85 – 89	80 – 84	< 80	ER visit or hospitalization due to hypotensive shock
Respiratory rate – breaths per minute	17 – 20	21 – 25	> 25	Intubation

Patients should be at rest during vital sign measurements.
Oral temperature; no recent consumption of hot or cold drinks or smoking.
***Heart rate between 60 – 100 bpm. Consider baseline bradycardia for non-athletes during clinical assessment.**

Table 3. Severity of Systemic (General) Adverse Events
Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)

Systemic (General) Event	Grade 1	Grade 2	Grade 3	Grade 4
Nausea	No interference with activity or 1–2 episodes per 24 hours	Some interference with activity or > 2 episodes per 24 hours	Prevents daily activity, requires outpatient IV hydration	ER visit or hospitalization for hypovolemic shock
Vomiting	No interference with activity or 1–2 episodes per 24 hours	Some interference with activity or > 2 episodes per 24 hours	Prevents daily activity, requires outpatient IV hydration	ER visit or hospitalization for hypovolemic shock
Diarrhea	2–3 soft stools or < 400 g per 24 hours	4–5 stools or 400–800 g per 24 hours	6 or more liquid stools or > 800 g per 24 hours, or requires outpatient IV hydration	ER visit or hospitalization
Headache	No interference with activity	Repeated use of non-opioid analgesics > 24 hours or some interference	Significant; use of any opioid analgesic or prevents daily activity	ER visit or hospitalization
Fatigue	No interference with activity	Some interference with activity	Significant; prevents daily activity	ER visit or hospitalization
Myalgia	No interference with activity	Some interference with activity	Significant; prevents daily activity	ER visit or hospitalization

Table 4. Severity of Symptoms Related to Rheumatic Fever for Detecting Adverse Events
Adapted from CTCAE version 5.0

Systemic (Related to Rheumatic Fever)	Grade 1	Grade 2	Grade 3	Grade 4
Euphoria	Mild mood elevation	Moderate mood elevation	Severe mood elevation (e.g., hypomania)	–
Depression	Mild depressive symptoms	Moderate depressive symptoms; limitation of daily instrumental activities	Severe depressive symptoms; limitation of self-care activities; hospitalization not indicated	Life-threatening, self-harm or harm to others; hospitalization indicated
Arthralgia	Mild pain	Moderate pain; limitation of daily instrumental activities	Severe pain; limitation of daily self-care activities	–
Dyspnea	Shortness of breath with moderate exertion	Shortness of breath with minimal effort; limitation of daily instrumental activities	Resting shortness of breath; limitation of self-care activities	Life-threatening; urgent intervention indicated
Rash	Macules/papules covering <10% body surface area, with or without symptoms (e.g., pruritus, burning, tightness)	Macules/papules covering 10–30% body surface area, with or without symptoms; limitation of daily instrumental activities	Macules/papules covering >30% body surface area, with or without associated symptoms; limitation of self-care activities	–
Salivary gland enlargement	Asymptomatic; only clinical or diagnostic observations; intervention not indicated	Moderate symptoms; limitation of daily instrumental activities	Severe symptoms; limitation of self-care activities; intervention indicated	–
Chest pain (cardiac)	Mild pain	Moderate pain; limitation of daily activity	Pain at rest; limitation of self-care activities	–
Heart murmur	–	Asymptomatic	Symptomatic; symptoms controlled with medical intervention	Life-threatening; urgent intervention indicated
Abnormal heart	–	Asymptomatic	Symptomatic; symptoms	Life-threatening;

Systemic (Related to Rheumatic Fever)	Grade 1	Grade 2	Grade 3	Grade 4
sounds (including third heart sound)			controlled with medical intervention	urgent intervention indicated
Palpitations	Mild symptoms; no intervention indicated	Intervention indicated	–	–
Pre-syncope	–	No intervention needed (e.g., near fainting)	–	–
Syncope	–	–	Orthostatic collapse with fainting	–
Dyskinesia	Mild involuntary movements	Moderate involuntary movements; limitation of daily instrumental activities	Severe involuntary movements or torticollis; limitation of daily self-care	Life-threatening; urgent intervention indicated
Seizures	Brief partial seizures without loss of consciousness	Brief generalized seizures	Multiple seizures despite medical intervention	Life-threatening; prolonged recurrent seizures
Erythema marginatum	–	Erythema covering >90% of body surface without symptoms	Erythema covering >90% of body surface with symptoms (e.g., pruritus or sensitivity)	–
Osler nodes	–	No intervention needed	Hindering daily activity and requiring medical intervention	–

Table 5. Severity of Serological Abnormalities Classified as Adverse Events
Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)

Serum Parameter *	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4 ****
Blood Urea Nitrogen (BUN) mg/dL	23–26	27–31	> 31	> 66.4 (requires dialysis)
Serum Creatinine (mg/dL)	1.5–1.7	1.8–2.0	2.1–2.5	> 2.5 (requires dialysis)
CPK (mg/dL)	1.25–1.5× ULN***	1.6–3.0× ULN	3.1–10× ULN	>10× ULN
Liver function tests (ALT, AST) enzyme increase	1.1–2.5× ULN	2.6–5.0× ULN	5.1–10× ULN	>10× ULN
Bilirubin (when elevated with liver enzyme increase)	1.1–1.25× ULN	1.26–1.5× ULN	1.51–1.75× ULN	>1.75× ULN
Bilirubin (normal liver tests but elevated)	1.1–1.5× ULN	1.6–2.0× ULN	2.0–3.0× ULN	>3.0× ULN
Pancreatic enzymes (Amylase)	1.1–1.5× ULN	1.6–2.0× ULN	2.1–5.0× ULN	>5.0× ULN
C-reactive protein (mg/L)	1–3	3.1–10	>10	–

Note:
Results at the borderline of Grade 1 are not considered adverse events if within the laboratory's normal reference range. Clinical signs or symptoms associated with laboratory abnormalities may classify the abnormality as potentially life-threatening (Grade 4). For example, hyponatremia within Grade 3 (125-129 mEq/L) is considered Grade 4 if associated with new seizure activity.
ULN = upper limit of normal

Table 6. Severity of Hematological Laboratory Alterations to Be Classified as Adverse Events *(Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)*

Hematology *	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Hemoglobin (Women) - g/dL	11.0 – 12.0	9.5 – 10.9	8.0 – 9.4	< 8.0
Hemoglobin (Women) — Baseline value change - g/dL	Any decrease up to 1.5	1.6 – 2.0	2.1 – 5.0	> 5.0
Hemoglobin (Men) - g/dL	12.5 – 13.5	10.5 – 12.4	8.5 – 10.4	< 8.5
Hemoglobin (Men) — Baseline value change - g/dL	Any decrease up to 1.5	1.6 – 2.0	2.1 – 5.0	> 5.0
Increase in leukocytes - cells/mm³	10,800 – 15,000	15,001 – 20,000	20,001 – 25,000	> 25,000
Decrease in leukocytes - cells/mm³	2,500 – 3,500	1,500 – 2,499	1,000 – 1,499	< 1,000
Decrease in lymphocytes - cells/mm³	750 – 1,000	500 – 749	250 – 499	< 250
Decrease in neutrophils - cells/mm³	1,500 – 2,000	1,000 – 1,499	500 – 999	< 500
Eosinophils - cells/mm³	650 – 1,500	1,501 – 5,000	> 5,000	Hypereosinophilia
Decrease in platelets - cells/mm³	125,000 – 140,000	100,000 – 124,000	25,000 – 99,000	< 25,000

Hematology *	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Erythrocyte sedimentation rate (adults under 50 years, men) – mm/h	16–50	51–90	> 90	–
Erythrocyte sedimentation rate (adults under 50 years, women) – mm/h	21–50	51–90	> 90	–

Note:
A result at the borderline of Grade 1 will not be considered an adverse event if within the normal laboratory range of the institution.

Table 7. Severity of Urinary Laboratory Alterations to Be Classified as Adverse Events
Adapted from the toxicity grading scale for healthy adult and adolescent volunteers enrolled in preventive vaccine clinical trials (U.S. Department of Health and Human Services, 2007)

Urine *	**Grade 1**	**Grade 2**	**Grade 3**	**Grade 4**
Protein	Trace	1+	2+	Hospitalization or dialysis
Glucose	Trace	1+	2+	Hospitalization due to hyperglycemia
Blood (microscopy) (red blood cells per field)	1–10	11–50	>50 and/or gross blood	Hospitalization or transfusion of red cell concentrate

Note:
A result at the borderline of Grade 1 will not be considered an adverse event if within the normal laboratory range of the institution.

Table 8. Severity of Selected Cardiac Disorders to Be Classified as Adverse Events
Adapted from CTCAE version 5.0

Cardiac Disorders	Grade 1	Grade 2	Grade 3	Grade 4
Increased PR interval	Asymptomatic, intervention not indicated	Non-urgent intervention indicated		
Complete atrioventricular block		Non-urgent intervention indicated	Incomplete symptomatic block medicated or controlled with device (e.g., pacemaker)	Life-threatening; urgent intervention indicated
Corrected QT interval (QTc) prolonged on ECG	450–480 ms	481–500 ms	≥501 ms on at least two ECGs	≥501 ms or >60 ms change from baseline with “Torsades de pointes” or polymorphic ventricular tachycardia or signs/symptoms of severe arrhythmia
Aortic valve disease	Asymptomatic, valvular thickening with or without mild regurgitation or stenosis on imaging			
Mitral valve disease	Asymptomatic, valvular thickening with or without mild regurgitation or stenosis on imaging	Asymptomatic, moderate regurgitation or stenosis on imaging	Symptomatic, severe regurgitation or stenosis on imaging; symptoms controlled with medical intervention	Life-threatening; urgent intervention (e.g., valve replacement, valvuloplasty)
Pulmonary valve disease	Asymptomatic, valvular thickening with or without mild regurgitation or stenosis on imaging	Asymptomatic, moderate regurgitation or stenosis on imaging	Symptomatic, severe regurgitation or stenosis on imaging; symptoms controlled with medical intervention	Life-threatening; urgent intervention (e.g., valve replacement, valvuloplasty)

Cardiac Disorders	Grade 1	Grade 2	Grade 3	Grade 4
Tricuspid valve disease	Asymptomatic, valvular thickening, with or without mild regurgitation or stenosis on imaging	Asymptomatic; moderate regurgitation or stenosis by imaging	Symptomatic, severe regurgitation or stenosis on imaging; symptoms controlled with medical intervention	Life-threatening; urgent intervention (e.g., valve replacement, valvuloplasty)
Myocarditis	Asymptomatic with cardiac changes on imaging	Symptoms with moderate to severe activity or exertion	Severe symptoms at rest or with minimal activity/exertion; intervention indicated	Life-threatening; urgent intervention (e.g., continuous IV therapy or mechanical hemodynamic support)

Adverse events at the injection site (Table 1) will be monitored up to 1 week after each dose. Vital signs and systemic adverse events, as well as symptoms related to RF (Tables 2, 3, and 4), will be actively assessed at all study visits. Laboratory investigations (Tables 5, 6, and 7) will be performed in weeks 2 and 4 after each dose, as well as during the final visit of the study, 26 weeks after the last dose. Electrocardiography and echocardiography will be conducted in week 8 following doses 2 and 3 and at the end of the study to detect cardiac disorders (Table 8). Safety assessments are summarized in Table 9. The severity of adverse events will be classified according to the above-mentioned tables. For adverse events not described in this section, the toxicity grading scale for healthy adults and adolescent volunteers involved in preventive vaccine trials, developed by the FDA in 2007 (U.S. Department of Health and Human Services, 2007), should be followed.

5.5.2.2 Immunosurveillance

Serum samples will be evaluated for cross-reactivity with peptides from human cardiac myosin previously identified as potential targets of autoimmune reactions. High-binding 96-well plates will be pre-coated with human cardiac myosin peptides in carbonate buffer for 1 hour at 37°C. After washing with PBS-Tween 20 and blocking with PBS-BSA for 1 hour at 37°C, the serum samples from study participants will be added in PBS-BSA buffer and incubated for 18 hours at 4°C. Following washing, the plates will be incubated with human anti-IgG conjugated to peroxidase for 1 hour at 37°C. After another wash, the OPD substrate will be added for 30 minutes at 37°C, and the reaction will be stopped with H_2SO_4. Absorbance will be read using an automated plate reader at 490 nm.
Antibodies against SLO (streptolysin O), anti-DNAse B, and rheumatoid factor will also be tested.

5.5.2.3 Hypersensitivity Reactions

Considering the possibility of hypersensitivity reactions to the vaccine, the induction of total and specific IgE antibodies against the peptide will

be evaluated in participants before and after immunizations, since this reaction type is mediated by IgE. Detection will be performed via ELISA, using a method similar to that described in item 5.4.2.2., but employing anti-IgE conjugated to peroxidase.

A skin prick test will also be performed to assess the potential development of hypersensitivity reactions before immunizations. This test involves skin exposure to the StreptInCor vaccine antigen (peptide) via pricking, and the response is measured by redness, itching, or swelling (wheal) at the site after 15 minutes.

A member of our medical team specialized in adverse drug reactions (clinical allergist) will be present to closely monitor and manage any immediate hypersensitivity reactions during the 2 hours following immunization. If any participant shows an immediate hypersensitivity reaction, serum tryptase will be collected within two hours of symptom onset and compared to baseline levels.

5.5.3 Surveillance and Reporting of Adverse Events

Adverse events must be recorded in the participants' medical records from the administration of the first dose of the study product. All participants will be instructed to contact the study team if adverse events occur. Responses to structured safety interviews and all adverse events will be routinely reported to the principal investigator through medical records. Reported events should include their clinical outcome (recovery, stabilization, sequelae, or death).

Serious adverse events (SAEs) are defined as any undesirable medical occurrence, at any dose, that:
a) results in death;
b) is life-threatening;
c) requires or prolongs hospitalization;
d) results in persistent or significant disability, or predisposes to a congenital anomaly.

All SAEs, regardless of causal relation to the study product, must be reported to the study responsible within 24 hours of awareness. The study team is also responsible for reporting SAEs to regulatory agencies and ethics committees, in accordance with current legislation. The outcomes of events must be reported as soon as the investigators become aware of them. Outcome reports should be ongoing throughout the study and not restricted to specific visits.

5.5.4. Safety Evaluation Period

The safety evaluation period will start immediately after the first dose and end six months after the last dose. Events ongoing at the end of this period will be followed to determine their causal relationship and outcome.

Any female participant who becomes pregnant during the study will be monitored until delivery to assess potential congenital anomalies or birth defects with a reasonable causal relationship to the study product.

All adverse events will be recorded with their type, duration, and severity.

5.6 Criteria for Withdrawal of a Participant from the Study Protocol
A participant may be withdrawn if:

- He or she requires or uses prohibited medication;
- She becomes pregnant (female participants);
- She requests discontinuation from the study or withdraws informed consent.

A physician may also withdraw a participant in cases of Grade 2 or higher adverse events with a reasonable causal relation to the study product, or if any unresolved condition could interfere with study procedures.

All participants withdrawn from the study product will be discontinued once the safety evaluation period is over.

5.7 Criteria for Discontinuation of a Participant from the Study
A participant will be withdrawn upon their own request. Such participants will be encouraged to remain in safety follow-up without receiving new doses of the study product. If the participant refuses safety assessments, they will be invited for a withdrawal consultation, following the same procedures as the final study visit (Visit 14), as shown in Table 9.

5.8 Criteria for Suspension or Termination of the Study
The study may be suspended if at least:

- Five individuals experience Grade 2 or higher adverse events with a reasonable causal relationship to the study product;
- Three individuals experience Grade 3 or higher adverse events with a reasonable causal relationship;
- One individual experiences Grade 4 adverse events with a reasonable causal relationship;

- One individual shows signs of RF-related symptoms at Grade 2 or higher (Table 4);
- One individual shows a Grade 2 or higher cardiac disorder (Table 8);
- One individual has cross-reactive antibodies against cardiac myosin peptides.

In such cases, an independent ad hoc committee may be convened to advise on whether to terminate or continue the study.

The study can also be suspended or canceled at the request of regulatory or ethics authorities or if the sponsor chooses to do so based on new scientific information.

Figure 6. Study Procedures Flowchart

Table 9. Study Procedures Flowchart

Visit	**C1**	**C2**	**C3**	**C4**	**C5**	**C6**	**C7**	**C8**	**C9**	**C10**	**C11**	**C12**	**C13**	**C14**
Procedure	Screening	D1	D1+2 (±3d)	D2 (D1+4 ±3d)	D2+2 (±3d)	D2+4 (±5d)	D2+8 (±7d)	D2+18 (±7d)	D3 (D1+26 ±7d)	D3+2 (±3d)	D3+4 (±5d)	D3+8 (±7d)	D3+18 (±7d)	D3+26 (±14d)
Procedures														
Informed Consent	X													
Randomization		X												
Vaccination			X											
Clinical Evaluation		X	X	X	X	X	X	X	X	X	X	X	X	X
Cardiology Assessment		X	X	X	X	X	X	X	X	X	X	X	X	X
Neurological Assessment			X											
Adverse Events			X	X	X	X	X	X	X	X	X	X		
Hemogram		X	X	X	X	X		X	X	X				
ESR		X	X	X	X	X		X	X	X				
Creatine Kinase (CK)		X	X	X	X	X		X	X	X				
TGO		X	X	X	X	X		X	X	X				
TGP		X	X	X	X	X		X	X	X				
Bilirubin		X	X	X	X	X		X	X	X				
Urea		X	X	X	X	X		X	X	X				
Creatinine		X	X	X	X	X		X	X	X				
Tryptase		X	X	X	X	X		X	X	X				

Visit	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12	C13	C14
Amylase		X	X	X	X	X	X	X	X					
Anti-HIV		X	X	X	X	X	X	X	X					
HBsAg		X	X	X	X	X	X	X	X					
Anti-HBs		X	X	X	X	X	X	X	X					
Anti-HCV		X	X	X	X	X	X	X	X					
Urinalysis		X	X	X	X	X	X	X	X					
β-hCG (urine)*		X					X							
C-reactive protein		X	X	X	X	X	X	X	X					
ASLO		X	X	X	X	X	X	X	X					
Anti-DNAse B		X	X	X	X	X	X	X	X					
Troponin		X	X	X	X	X	X	X	X					
ANA (FAN)		X	X	X	X	X	X	X	X					
Rheumatoid Factor		X	X	X	X	X	X	X	X					
Complement		X	X	X	X									

Table 10. Immunological Tests

Test	Method and Interpretation	Sample	Purpose
Detection of antigen-specific humoral immune response	Detection of antibodies by ELISA: increase in antibody levels compared to pre-vaccination levels	0.5 mL serum (whole blood)	Immunogenicity
Antibody binding to bacterial surface	Flow cytometry detection: correlation with negative and positive controls	0.5 mL serum (whole blood)	Functionality
Streptococcal invasion/adhesion	Bacterial plaque assay: percentage of invasion/adhesion based on CFU counts before and after serum treatment	0.5 mL serum (whole blood)	Functionality
Cell proliferation	Flow cytometry detection of cell proliferation after stimulation with the vaccine peptide: correlation with negative and positive controls	20 mL whole blood	Immunogenicity
Cytokine detection	Cytometric Bead Array (CBA): comparison with known concentration standards	0.5 mL serum (whole blood)	Immunogenicity
Opsonophagocytosis	Bacterial plaque assay: percentage of phagocytosis based on CFU counts in controls	1 mL serum (whole blood)	Functionality
HLA Class II typing	Standard clinical-laboratory procedure: genotypic description	200 ng DNA (whole blood)	Genetic inheritance
Cross-reactive antibody detection	ELISA: OD below the pre-established cutoff value	1 mL serum (whole blood)	Safety

Abbreviations:
ELISA: Enzyme-Linked Immunosorbent Assay; OD: optical density; CFU: colony-forming units; CBA: Cytometric Bead Array.

"8. Study Schedule"

"Table 11. Study Schedule"

Month	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35
Volunteer Screening	X																																		
Step 1 Vaccination 1		X																																	
Step 1 Vaccination 2			X																																
CMDS Meeting				X																															
Step 1 Vaccination 3								X																											
CMDS Meeting									X																										
Step 2 Vaccination 1									X																										
Step 2 Vaccination 2										X																									
CMDS Meeting											X																								
Final Safety Assessment Step 1														X																					
Step 2 Vaccination 3															X																				
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Final Safety Assessment Step 2																					X														
Step 3 Vaccination 3																						X													
CMDS Meeting																							X												
Step 4 Vaccination 1																							X												
Step 4 Vaccination 2																								X											
CMDS Meeting																									X										
Final Safety Assessment Step 3																												X							
Step 4 Vaccination 3																													X						
Final Safety Assessment Step 4																																			X

9. Ethical Considerations

9.1 Description of possible physical, social, and psychological risks and methods to minimize them

Potential risks may be observed after participation in clinical trials, particularly vaccination. All potential participants will receive comprehensive explanations about the protocol procedures. The Research Ethics Committee (REC) will ensure that the study is conducted following high standards of safety and ethics. As previously guaranteed, all possible risks and benefits will be explained to each interested volunteer during the informed consent process. All vaccine candidates are extensively tested before entering human clinical trials; however, adverse events or reactions caused by the vaccine may still occur. These are often mild and may include fever and local inflammation at the injection site. These effects must be clearly explained to all volunteers during informed consent. It is also critical that volunteers understand there is a possibility that the vaccine under study may be ineffective or that they might be randomly assigned at the start of the trial to receive a placebo, an inert substance. Regardless, participation does not necessarily protect against FRA. Other potential risks include psychological and social issues triggered by the possible negative social stigma related to participation in clinical trials. Nonetheless, this study does not present other relevant psychological or social risks requiring special mention in the protocol.
Finally, the most significant adverse reaction is the possibility of developing a disease or syndrome similar to FRA, with severe symptoms such as new heart murmurs, arrhythmias, permanent valve disease, myocarditis, arthritis, neurological conditions like seizures or chorea, as well as other conditions described in Tables 4 and 8 of the possible adverse events section.

We emphasize that participants will have the right to free comprehensive assistance for any direct/indirect, immediate/late damages, for as long as necessary, guaranteed by the sponsor, as per items II.3.1 and II.3.2 of CNS Resolution No. 466/2012. The study may be interrupted, with prior approval from the REC, whenever deemed necessary to safeguard the participant due to adverse effects or other pertinent situations (CNS Resolution No. 251/1997, item III.2.e). At the end of the study, all participants will be followed up at the Heart Valve Clinic of the Heart Institute-HCFMUSP for an additional 12 months, or longer if deemed necessary, to increase safety against possible late adverse effects related to the study medication.

Despite these inherent risks, the researchers have taken all measures to ensure the safety and ethics of this vaccination trial and that it can contribute to overall community health. Several in vitro immune tests will be performed to ensure that the vaccine does not induce autoimmune reactivity.

9.2 Anticipated benefits

There are no expected benefits for the study participant through the use of the investigational product. Participants will receive free results of all safety assessments.

9.3 Implementation and documentation of free and informed consent

Informed consent will be offered to potential study participants before any procedures are conducted. The consent will be documented in the form approved by the respective ethics committee. The consent form and documentation must comply with the requirements of CNS Resolution No. 466/2012 and Good Clinical Practice standards.

9.4 Protection of privacy and confidentiality

All study-related documents containing personally identifiable information must be stored in a locked cabinet with restricted access to the study team. Case report forms, adverse event reports, and other information sent to the study monitor cannot contain any identifiers of participants. It is important that documents are anonymized by blacking out personal identification before being forwarded by the medical team responsible for participant care to any other entity (items III.2i and IV.3e of CNS Resolution No. 466/2012). Each participant will be assigned a protocol number used to maintain anonymity.

Monitors, auditors, inspectors, or representatives of regulatory and ethics authorities may access identifying documentation, and this must be clearly indicated in the informed consent.

9.5 Reimbursement and incentives

Volunteers will have their transportation expenses reimbursed each time they attend the study site. A light meal with a snack and drink may be provided after sample collection. No payment will be made to participants.

9.6 Insurance

Volunteers are entitled to health insurance for serious adverse effects or development of FRA, and if necessary, compensation through specific insurance for this purpose.

10. Statistical Analysis and Data Handling

10.1 Data analysis plan

Descriptive statistics will be used to analyze safety and immunogenicity data. Each of the four phases will include three placebo recipients, plus twelve volunteers in each group: low dose, low-mid dose, mid-high dose, and high dose. Clinical and laboratory results will remain blinded.

10.2 Statistical analysis
Parametric and non-parametric tests will compare results in terms of immunological parameters before and after each dose. For all statistical tests, p-values less than 0.05 will be considered statistically significant with 80% power.

10.3 Data collection
Information will be collected via electronic case report forms designed for this purpose. Each authorized user will have an individual password, and the username/password combination will be considered equivalent to a team member's signature. No information can be directly recorded into electronic case report forms. The research center will maintain a source document where all information is registered. Only the randomized participant number will be recorded electronically, without any identifying information.

10.4 Data registration, storage, and archiving
Participant records at each institution, laboratory reports, notes, and reports are considered source documents for this study. Electronic case report forms are not source documents. Records and source documents must be stored in locked cabinets or restricted access rooms for 15 years after study completion. If a site cannot secure documents for 15 years, they must immediately notify the study responsible to ensure proper archiving.

10.5 Quality control/ assurance
Study sites are responsible for data quality and must implement quality control systems. The monitor will support these activities and conduct additional quality checks, including verification of source documents. The study leader may audit any stage of the study as part of quality control.

10.6 Interim reviews and analyses
No interim reviews or analyses are planned for this study.

10.7 Direct access to source data/documents
Direct access to source data/documents is guaranteed to monitors, auditors, inspectors, or regulatory and ethics representatives upon request. This includes participant records within the institution.

10.8 Regulatory statement
This study will be conducted following Brazil's CNS Resolution No. 196/96, the Declaration of Helsinki (2013 version), the Pan American Health Organization guidelines, ICH E6 Good Clinical Practice, and all applicable regulations of the Brazilian regulatory agency, ANVISA.

10.9 Publication policy

The study will be registered with the WHO International Clinical Trials Registry Platform. Results will be published in this registry and prepared for peer-reviewed journals and scientific meetings. The study lead must be notified at least two weeks before submission of results to any journal or conference. Results will also be disseminated in simple language to study participants.

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