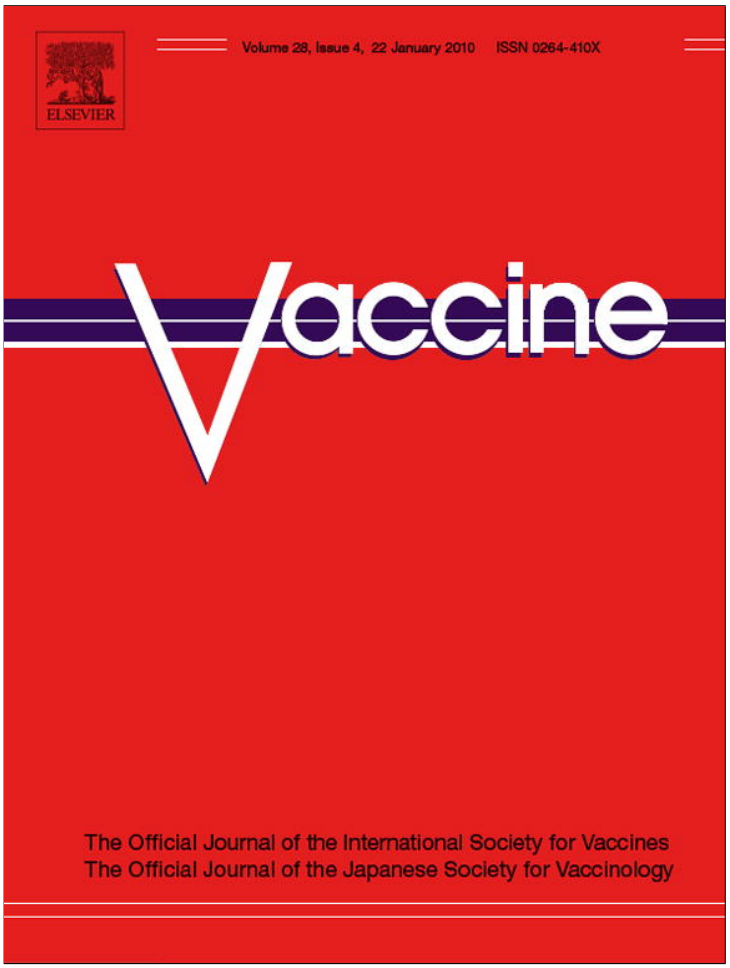

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Double-blind, randomized, placebo-controlled Phase I Clinical Trial of the therapeutical antituberculous vaccine RUTI®

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ABSTRACT

A Phase I interventional Clinical Trial was performed with a potential tuberculosis vaccine, based on detoxified cellular fragments of *M. tuberculosis*, named RUTI®. The objective was to evaluate the safety profile and T-cell immune responses over a 6-month period following subcutaneous inoculation. The double-blind, randomized and placebo-controlled trial was conducted in healthy volunteers, all recruited at one site. RUTI®, at each of the four tested doses, starting from 5 μg and going up to 200 μg, and placebo were inoculated to groups of 4 and 2 volunteers respectively, consecutively. RUTI® appeared to be well tolerated as judged by local and systemic clinical evaluation, though vaccine dose dependent local adverse reactions were recorded. T-cell responses of blood lymphocytes to PPD and a number of antigen subunits were elevated, when compared with controls subjects. These results support the feasibility of future evaluation, to be targeted at subjects with latent tuberculosis infection (LTBI).

1. Introduction

Tuberculosis is one of the most frequent infectious diseases in the world, in spite of being a curable disease [1]. The latency state of the *M. tuberculosis* contributes to that, as even without symptoms, latently infected individuals have the possibility to develop active tuberculosis throughout their lives, with the spread of the disease being implied. Unfortunately, only indirect immunological methods are able to detect the presence of latent bacilli: the tuberculin skin test (TST) and T-cell interferon-gamma release assays (TIGRA) used in LTBI diagnostic [2]. LTBI is treated in order to prevent the progression to an active TB, achieving a 90% decrease in the incidence of TB in TST converters if treated with isoniazid for 9 months [3]. But this treatment is too long, does not ensure the complete cure of the patient and it is not exempt of adverse effects and its compliance becoming difficult. Research on latent tuberculosis has focused on the development of prophylactic vaccines and of new therapeutical regimens, but the 9-month treatment with INH is still the best approach in terms of cost-efficiency [4]. After being avoided since the mid 20th century, for the fear of its adverse effects (Koch's reaction), the therapeutical vaccination to treat tuberculosis has been reintroduced. More than 100 years later, a new vaccine named RUTI®, based on detoxified and liposomed cellular fragments of *M. tuberculosis* bacilli (FCMtb) has been developed under good manufacturing practices (GMP) in Badalona (Catalonia, Spain), by Archivel Farma. Proposed as a part of a therapeutical regimen in combination with short-term chemotherapy (1 month with INH) and its safety being proven in several animal models and based on all the non-clinical development results, all the licences to start its clinical development were obtained.

The study presented here is a double-blind, randomized, placebo-controlled Phase I Clinical Trial (CT), whose objectives were to evaluate the tolerability and the Immunogenicity of 4 increasing doses of the vaccine RUTI® (5, 25, 100 and 200 μg of

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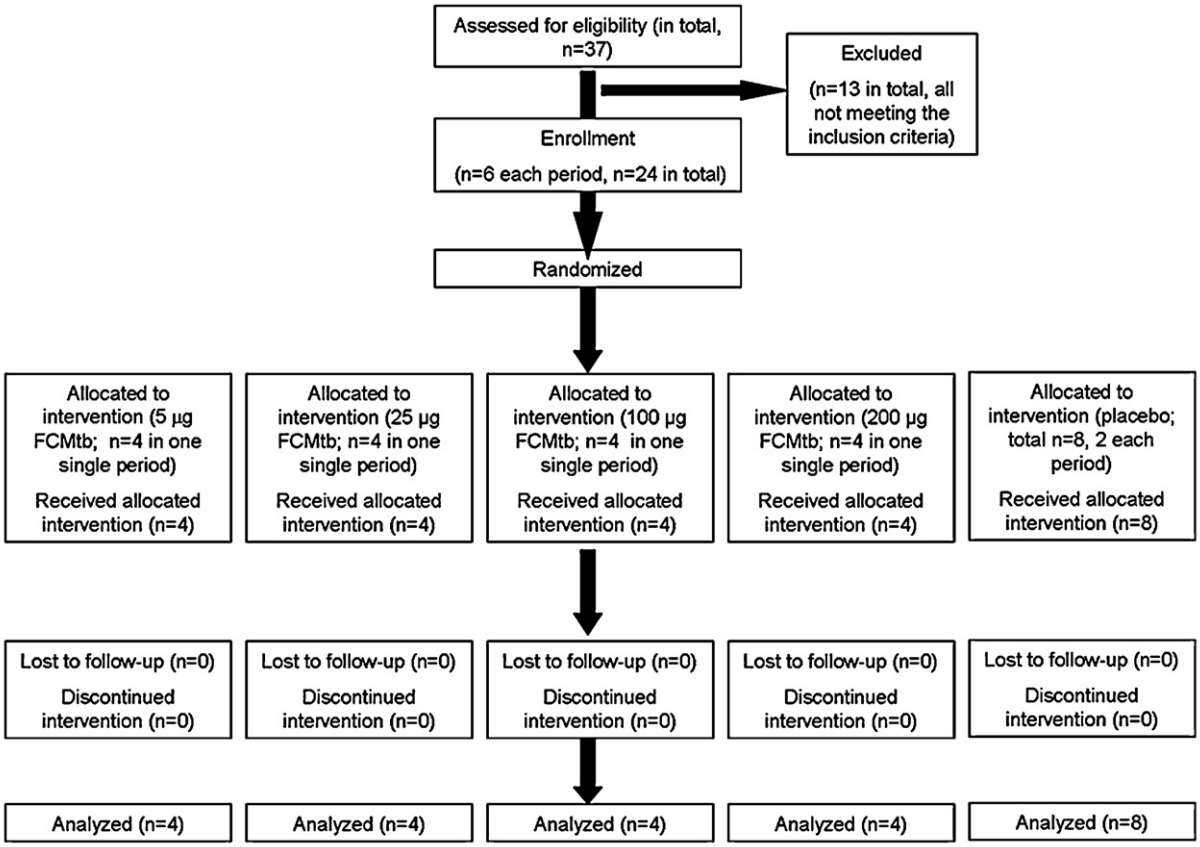

Fig. 1. Chart representing the participant flow of the CT. From 65 people interviewed, only 37 could be assessed for eligibility, enrolling 24 participants to participate in 4 different periods ($n = 6$ each period). All the 24/24 were randomized and allocated to intervention, receiving it, being fully followed-up, ending the trial and being analyzed.

FCMtb) administered to healthy volunteers twice (4 weeks apart) by the subcutaneous route.

2. Methods

2.1. Ethics

The protocol of the study was reviewed and approved by the Independent Ethics Committee (IEC) at the investigational centre (Hospital Universitari "Germans Trias i Pujol", Badalona, Catalonia, Spain), and the Member State Regulatory Authority (AEMPS, Spain). All investigators and collaborators agreed to rigorously observe Spanish deontological code, the Helsinki declaration with all its amendments, and the national guidelines respect to clinical trials conducted in humans, summarized in law 29/2006 (July 26th 2006. BOE no. 178) and the Royal Decree 223/2004 (February 6th 2004. BOE no. 33). The Good Clinical Practice guidelines of the ICH (International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use) were also followed. All the objectives, methodology as well as possible inconveniences and risks due to the study were explained to each subject, orally and in writing (by a consent form) before their inclusion. They were also informed of the different treatments to be tested, the way they would be assigned to the groups, the possible benefits of the study, their option to their own withdrawal at any time and of the existence of an insurance contract. All the selected volunteers signed the consent form before starting any procedure. The study protocol was reviewed and approved, being registered as EudraCT number: 2006-000690-29; PEI Code: 06-075 and ClinicalTrials.gov identifier: NCT00546273.

2.2. Participants

Subjects were interviewed, screened for enrolment in 4 sequential phases about 14 days before starting each period of the trial at the Phase I Unit of the Hospital Germans Trias i Pujol (Badalona, Catalonia, Spain) by the physicians of the Unit. A total of 65 men were interviewed during the whole 4 recruiting periods. After signing the informed consent, 37 of them were screened in order to evaluate the inclusion/exclusion criteria (Fig. 1). All white men aged 18–40 complying with all the requirements at the moment of screening: (1) normal physical exam; (2) body mass index between 17 and 29.9 kg/m^2; (3) systolic blood pressure between 100 and 140 mmHg; (4) diastolic blood pressure between 50 and 90 mmHg; (5) heart rate between 50 and 90 beats/min, measured in supinus decubitus after 3 min of resting in this position; (6) axillary corporal temperature <37 °C; (7) ECG without clinically relevant signs. All those able to communicate fluently with the investigators and to fulfil all the trial conditions were candidates for inclusion in the study. Subjects were not included in the study if any of the following criteria were present: (1) antecedents or actual evidence of severe diseases that could be relevant to the study, including autoimmunity disorders, severe allergy or any chronic or seasonal allergy; any severe adverse event or hypersensibility to any drug or vaccine or contraindication to the excipients used in the vaccine to be tested; (2) drug abuse or addiction (including an alcohol intake >50 g per day), or mental handicap; (3) acute disease with body temperature >37 °C 72 h before the inoculation of the vaccine to be tested; (4) T-SPOT TB positive result; (5) the presence of Antinuclear Autoantibodies (ANA); (6) previous vaccination with BCG; (7) image compatible with pulmonary tuberculosis infection in the chest radiological exam; (8) having had any investigational drug

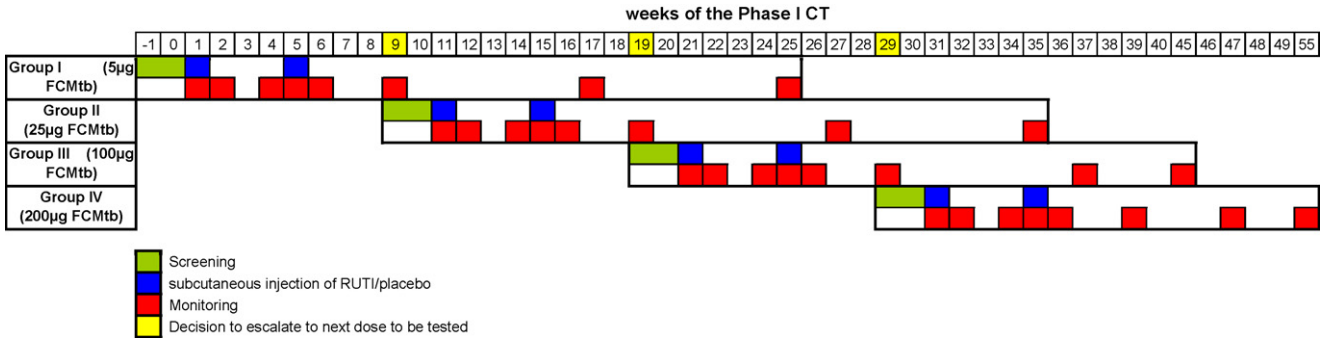

Fig. 2. Study design, with the screening phases, interventions, monitoring and decision to escalate to next dose to be tested. This chart should be useful to understand how the increasing doses of the vaccine were sequentially enchained, beginning once the previous dose safety ensured. It also helps to see when the follow-up visits were made.

during the previous month to the first inoculation of the experimental vaccine to be tested in this study; (9) loss of >400 mL of blood in the previous 12 weeks or >250 mL in the previous 4 weeks before screening; (10) any clinically relevant deviation of normality detected in the screening; (11) any relevant laboratory parameter abnormality detected in the screening; (12) positive results for antigen HBsAg or antibody IgM Anti-HBc of Hepatitis B virus (VHB), antibodies against Hepatitis C virus (VHC) or antibodies against HIV, detected in the screening; (13) intake of any drug that could not be eliminated from the organism before the first inoculation of the experimental drug to be tested (especially NSAIDs and corticosteroids); or (14) impossibility of obtaining biological samples.

A total of 13 volunteers were excluded because they did not fulfil the study's requirements. The other 24 volunteers gave their written informed consent to participate and also to be included in a local government database to control the participation of healthy volunteers in clinical trials. Before their inclusion in one of the periods, an appointment for the screening visit was made for each volunteer where a complete anamnesis, a complete physical exam, an ECG, a radiological chest exam and a laboratory parameters exam (haemato-biochemical parameters, T-SPOT TB, Antinuclear Autoantibodies (ANA), urinalysis and serology against HIV, VHB and VHC) were performed. All the results and data obtained from the volunteers during the whole study are considered confidential. Furthermore, all the investigators team certify that to their knowledge all subjects were healthy and thus considered able to be included in the CT, in terms of fulfilling all the study's requirements.

2.3. Interventions

A total of 4 periods (with 6 new volunteers each) were established sequentially in the study, in order to study 4 increasing doses of the vaccine (Fig. 2). Any period was only initiated once the safety of the previous one was ensured 4 weeks after the last individual was inoculated with the previous dose, considering this time lapse enough to observe any possible adverse event (AE). Before starting any of the 4 periods, and once the informed consent was obtained and the screening process finished, the 6 volunteers destined to enter this period were randomized to receive either placebo ($n = 2$) or the vaccine ($n = 4$). RUTI® was supplied by the sponsor and manufacturer (Archivel Farma s.l., Badalona, Catalonia, Spain) as an injectable vial with lyophilized product to be administered as a suspension by subcutaneous route. RUTI® is the name of the finished product, composed of the active ingredient of the vaccine named FCMtb (for detoxified cellular fragments of *M. tuberculosis*) plus excipients. The manufacturing process of RUTI® (under good manufacturing practice) has been published elsewhere [5–7]. Placebo was identically supplied and formulated except that it did not contain the active ingredient (FCMtb). Only a single batch of both RUTI® and placebo were used in the whole study. The distribution of the drug vials for the CT was carried out by the Pharmacy Department of Hospital Universitari Germans Trias i Pujol (Badalona, Catalonia, Spain) where the entire study drug received was inventoried and accounted for throughout the study. All the vials were manipulated on the day of administration in an identical way to achieve the prefixed dilution for each dose level. Both RUTI® and placebo were administered subcutaneously twice (28 days apart) by a qualified nurse of the Phase I Unit of the Pharmacology Department, supervised by the investigators and following the random plan specified in the study protocol. The inoculations were performed early in the morning, under fasting, and the exact time of administration for each volunteer was registered on the correspondent Case Report Form (CRF). The volunteers remained in the unit for a total of 3 h, and were evaluated for safety aspects at hour 3.

2.4. Objectives

The main objective of the present study was to evaluate the safety and tolerability and to establish a safety dosage range, if possible of RUTI®. The secondary one was to test the ability of the vaccine to trigger *M. tuberculosis* specific immune response in healthy people, compared to placebo. For this last purpose, and due to the lack of any correlation of protection for tuberculosis, several assays were conducted in order to help determine the most cost-effective techniques to evaluate RUTI®'s efficacy when designing a future Phase II CT.

Both objectives had the aim of demonstrating RUTI® to be safe and immunogenic in its first use in humans.

2.5. Outcomes

After the administration of each inoculation, each volunteer was followed-up during 25 weeks. The monitoring plan (Fig. S1) shows every procedure of the follow-up planned in the former study protocol and strictly followed during the study.

The volunteers were monitored by the investigators team of the Phase I Unit in order to perform the following safety evaluations: (1) record of both local and systemic adverse events, these being reported by the subject for the duration of the study both spontaneously and after questioning; (2) vital constants; (3) physical examination; (4) ECG and (5) laboratory safety tests. The complete Safety Assessments are listed in Table S1.

Adverse events detected by the investigator through interrogation or reported by the subject during the defined period of collection of AE were recorded. They were compiled and listed following the System Organ Class of MedDRA, considering an AE as any unwanted medical event in a subject in a clinical trial, regardless of its relationship with the intervention under evaluation; a serious AE defined as being cause of death, life threatening, requiring inpatient hospitalization, producing disability or incapacity persistent

or significant or threatening the patient. The investigator determined the relationship between the study medication and the AE as 'none', 'unlikely', 'possibly', 'probably', and 'not evaluable' according to a predefined algorithm based on the modified Karch and Lasagna algorithm used by the Spanish Pharmacovigilance System [8,9].

A VAS Pain score (visual analogical scale ranging from 0 to 100) was used to evaluate each volunteer subjective pain intensity at the inoculation point.

A total of 81 mL of blood was extracted from the volunteers by the nurse of the Phase I Unit, under fasting conditions and before any other procedure was done, at the timepoints indicated in the monitoring plan. The samples were properly labelled and sent to the central laboratories for laboratory safety testing (15 mL) and to the Experimental Tuberculosis Unit (UTE) (66 mL) for the Immunogenicity testing as soon as possible. The departments involved in the safety laboratory results (Haematology and Clinical Analysis, Immunology and Microbiology) as well as the UTE, are accredited by ISO procedures. The Clinical Research Organisation of the study monitored all the study in order to ensure the use of standard terminology and the collection of accurate, consistent, complete and reliable data.

The reliability of the Immunogenicity data was also ensured by the performance of all the assays by always the same technician (previously trained during a 6-month period) under the supervision of the former responsible for the Immunogenicity evaluation and analysis of all the results obtained. Accuracy of the performance of the techniques as well as the values obtained were ensured by following strict specific procedures (Standard Operating Procedures SOPs) that were previously designed and set up specifically for this CT and always under blinding premises. The sponsor also used an external auditing to review the following issues: data management and statistics, both the UTE and the Phase I Unit, source data/source documents and the Investigator File and the Trial Master File.

As long as no efficacy measurements are expected from a Phase I CT, nevertheless it was still important for the sponsor to determine whether the vaccine was or not able to generate any immune response against *M. tuberculosis* antigens in a healthy population and its Immunogenicity was evaluated (Table S2 and Supporting information). Most of the assays performed in this trial were based on previous bibliography that supported that the detection of interferon-gamma (IFN-γ) secretion was the most powerful tool to detect efficacy of any new drug against tuberculosis. IFN-γ is considered as the main tool that human beings have to fight tuberculosis [10,11] so this is the target for most of the assays performed in TB research. All the pre-clinical studies conducted with the vaccine RUTI® showed (as has been published elsewhere) that it is able to generate a strong cellular immune response measurable as IFN-γ secretion. In the same way, it has been previously used in the few other CTs that have been conducted with antituberculous vaccines [12–16] and has been included in the recently published panel of WHO recommendations for immunological outcomes of new tuberculosis vaccine trials [17]. In that sense, the IFN-γ secretion was measured by both commercial and non-commercial T-cell IFN-γ release assays (TIGRA) by two different techniques: the ELISPOT and ELISA, measuring two different concepts of the IFN-γ secretion, the number of the IFN-γ-secreting cells among a total of Peripheral Blood Mononuclear Cells (PBMC) and the total amount of IFN-γ secreted in a specific volume of whole blood, respectively [18]. The commercial tests used were T-SPOT TB (OxfordImmunotec, Oxfordshire, UK) and QuantiFeron-TB-Gold (Cellestis GmbH, Germany), both following the manufacturer's recommendations. The basis for using both commercial and non-commercial tests was that the commercial tests have been developed for diagnosing LTBI (latent tuberculosis infection), already validated to detect the presence of T effector cells against *M. tuberculosis* antigens secreted by growing bacilli [19,20]. Even though, the cut-off of both commercial techniques used were set up to diagnose people already infected, therefore not exactly valid to evaluate the cellular responses neither to a wider range of stimuli related to *M. tuberculosis* nor of healthy vaccinated people. The non-commercial tests where then used in order to improve the characterization of the immune response, searching for responses to up to 8 *M. tuberculosis* recombinant antigens, as well as BCG vaccine and PPD, both known as powerful stimuli for the immune response against *M. tuberculosis* (Table S3 and Supporting information). The measurement of T-Cellular populations by Flow Cytometry was only an overview to determine if the vaccine was able to enhance CD4 or CD8 T-cell populations. A total of 3 studies were performed with whole blood, based on published literature:

- The study of the secretion of IFN-γ by the γδ T lymphocytes was based on a paper published by Rojas et al. on 2005 in JID Rojas [21]. As γδ T lymphocytes secrete IFN-γ in response to phosphoantigens, as *M. tuberculosis* antigens are a phosphoantigen, the synthetic analogue bromohydrin pyrophosphate (BrHPP, kindly provided by Innate Pharma, France) was used to reproduce the experiment described in this paper. The goal was to elucidate if the vaccination with RUTI® was able to expand this cell subset when recognizing the synthetic phosphoantigen.
- The study of the mycobactericidal activity of the whole blood was based on "Bactericidal Activity in Whole Blood as a Potential Surrogate Marker of Immunity after Vaccination against Tuberculosis" published by Cheon et al. [22]. As the authors suggested that the mycobactericidal activity of the whole blood could be a surrogate marker of the immunity generated after an antituberculous vaccination, we reproduced one of the experiments described by Cheon et al.
- The third study using whole blood was a long-term whole blood assay, to test the supernatants of whole blood 7-days-cultures (with and without PPD) for IFN-γ by ELISA, and considering this response to be due to the Memory T-cells. In order to set up this assay, also previous published literature was used, and materials and methods adapted [23].

Whole blood samples of all the volunteers were sent to the Dr. Fréderic Altare (Institut de Pharmacologie et de Biologie Structurale, Toulouse, France) to be test for the study of the kinetic of granulomas formation around artificial beads coated with mycobacterial envelop compounds and around live mycobacteria.

Sera were collected to be tested for anti-*M. tuberculosis* antibodies (using both commercial tests and homemade ELISA assays, these in collaboration with Lionex Diagnostics and Therapeutics GmbH, Braunschweig, Germany) as well as for N alpha-Cbz-Lys-thiobenzylesteresterase (BLT-esterase) and granulolysin (both related to the host defense in human infectious disease) [24–26], in collaboration with Dr. Francesco Dieli, from the Dipartimento di Biopatologia e Metodologie Biomediche, Università di Palermo (Italy).

None of the Immunogenicity assessments performed in the present CT were standard procedures, as no marker exists to determine the reliable efficacy of an antituberculous vaccine. Different approaches exist in order to demonstrate superiority of the investigational drug to placebo. As no cut-off exists for those non-validated and research-only techniques, a statistical difference ($p < 0.05$) between the results of the different treatment groups has been considered enough to prove the superiority of RUTI® vs. placebo, or to consider one dose of FCMtb tested against the others. Considering 5 treatment groups (5, 25, 100 and 200 μg of FCMtb, and placebo), they have been compared with each other, as well as in terms of variability with respect the baseline results (those obtained at timepoint 0); its change between the different time-

points and their evolution through the whole follow-up period, by the statistical methods explained in Section 2.8.

2.6. Sample size

Given that this clinical test was an exploratory study of first administration in humans basically intending to demonstrate the safety of the vaccine, a formal predetermination of sample size based on numeric or statistic criteria was not made. In any case, the numbers shuffled in the test (24 subjects, 8 placebo and 16 of treatment) were within the standard for this type of studies. An independent monitoring safety committee – Data and Safety Monitoring Committee (DSMC) – was created to supervise the study and evaluate the safety of each dose by discussing the AE encountered until then. This committee met at week 9 of each period to decide whether to test a higher RUTI® dose or the need to stop the trial or withdraw any subject, as stated in the protocol.

2.7. Randomization and blinding

A person from Archivel Farma s.l., who was non-related to the CT, selected a random list chosen among 10 unpredictable allocation sequences generated using the Proc Plan of the SAS program. These data was used to prepare the medication and was kept locked secure place throughout the whole study until the disclosure of the blinding. Blind emergency code envelopes were prepared in three copies, one copy for the sponsor and two copies to be kept at site (at the Pharmacy and the Clinical Pharmacology Department). The envelopes were maintained in a locked secure place throughout the whole study while still allowing access for emergency code breaking only. Medication was conserved in the Pharmacy Department until its use.

2.8. Statistical methods

Results were expressed as mean and standard deviation (SD), frequencies and percentages (%), or as otherwise specified. All the analyses corresponding to the demographic variables, basal characteristics, as well as the variables to evaluate the Immunogenicity were made with the population per protocol (all randomized subjects who met the selection criteria, received the study medication, and did not present major protocol deviations). All the analyses corresponding to the security variables were carried out in the safety population (all patients who received at least one dose of the medication). The criteria for the definition of the study population were defined beforehand and the selection was performed during a Data Blind Review.

The immune response variables were assessed using a Mixed Models for Repeated Measurement (MMRM) approach [27], including baseline as a covariate and setting the variance covariance matrix to unstructured. The Fisher's exact test was used to compare categorical variables. Given the exploratory nature of this study of Phase I, no multiplicity adjustments were considered. The analysis was performed using SAS version 9.1.3 software (SAS Institute Inc., Cary, NC, USA) and the level of significance was established at the 0.05 level (two-sided).

3. Results

3.1. Participant flow and recruitment

The CT lasted 14 months (431 days) from the data the first patient enrolled to the time the last patient finished. After interviewing a total of 65 people during the whole CT, only 37 could be assessed for eligibility. A total of 13 volunteers were excluded at the screening because they did not fullfil the study's requirements: 1 showed sinechia pleuralis in his chest X-ray, 1 had a too higher corporal mass index, 1 had bradicardia (seen by the ECG), 1 had thrombocytopenia, 1 was a cannabis abuser, 1 was BCG vaccinated, 2 for antecedents of allergy, 2 of them had T-SPOT TB positive results and 3 for personal reasons. The screening was done in 4 phases, in the 14 days previous to starting each period of the CT. The 24 volunteers were enrolled to participate in one single period, each period composed of 4 volunteers vaccinated with RUTI® and 2 with placebo (total n allocated to intervention per period = 6), the subjects being randomized. As explained before, the 4 periods were started sequentially with the dose of FCMtb in the vaccine being the difference between them. All the volunteers (24/24) received the allocated intervention, were followed-up and ended the CT, with the results of all subsequently analyzed (Fig. 1).

No protocol deviations from the study as planned occurred.

Each volunteer was vaccinated with the allocated intervention (RUTI® or placebo) twice and followed-up for a total of 6 months. Each period was started with the screening of the subjects willing to participate after the decision to escalate to next dose to be tested had been made (in week 9 after starting the previous dose). Every subject included in the trial had the right to withdraw at any moment by immediately contacting the investigators to inform them, without obligation to provide reasons. This participant was supposed to be replaced by another except in the case the trial had to be ended because of safety issues, and would have received the corresponding medication that the one replaced would have received.

The DSMC was established to monitor strictly the study participants and to assess the progress of the clinical trial and the safety data. The DMSC convened at the predetermined intervals (week 9 of each period) to review the unblinded results of the volunteers already vaccinated, and it was this Committee's duty to decide whether to escalate to the next dose. No loss of follow-up or discontinuation of the treatment occurred, with the 24/24 volunteers being analyzed at the end of the CT in order to not break the blinding.

If a subject had withdrawn because an adverse event, he would have been followed-up by the investigators until resolution or reaching a clinically stable endpoint. The investigators had the right to decide to terminate or suspend the trial without prior agreement of the sponsor, by promptly informing the trial subjects (assuring them appropriate therapy and follow-up) the sponsor and the regulatory authority. None of the subjects included in the present CT decided to withdraw from the study, and none of them was removed by the investigators.

Fig. 2 summarizes these details in order to make the study design more comprehensible.

3.2. Baseline data

All demographic and clinical baseline characteristics for the 24 volunteers finally included in the CT with the corresponding standard deviations are summarized in Table 1. The anamnesis and physical exams performed by the volunteers during screening did not show any clinical significant abnormality. The ECG registers, as well as the results of the laboratory analysis of all the volunteers included in the study performed during the screening, were considered normal. Those values out of normal range by the laboratory were evaluated by the investigators considered to be without clinical significance.

3.3. Outcomes and estimation

Regarding the safety aspect of the study, results explained are divided into several subsections below.

Table 1
Demographic and cardiovascular baseline characteristics.

Parameter	Mean	SD	95% CI	Units
Age	24.08	3.46	22.62–25.55	years
Weight	72.60	10.56	68.14–77.06	kg
Height	1.78	0.06	1.76–1.81	m
Body mass index	22.57	2.45	21.54–23.61	kg/m^2
Systolic blood pressure	115.50	8.73	111.81–119.19	mmHg
Diastolic blood pressure	64.83	6.79	61.97–67.7	mmHg
Cardiac frequency	64.25	10.66	59.75–68.75	bpm
Corporal temperature	36.23	0.45	36.04–36.41	grades centigrades

The correspondent means, standard deviations, 95% CI and units are represented.

3.3.1. Results of laboratory tests

In general, the volunteers showed values within the limits of normality in the results of the clinical tests in the selection and visits throughout the study (blood count, biochemistry and urine analysis). Those results that were beyond the limits considered normal by the laboratory were evaluated by researchers as values not clinically significant, ongoing checks on those who could translate some type of pathology. The number of values that was outside the limits considered normal by the Laboratory of Clinical Analysis was 63, 16 of them were from haematology, 30 from biochemistry and 17 from urinalysis. A total of 60/63 had not clinical relevance; the other 3/63 had clinical relevance but was found not attributable to the investigational product.

3.3.2. Physical examination, ECG and vital signs

All the anomalies detected in the screening visit were not clinically significant. With regard to vital signs, practically all the values were within the limits of normality specified in the protocol, the deviations from normal values not being considered clinically significant. No ECG changes during the study were classified as clinically significant and in no case there were changes in the ECG that could be attributed to medication.

3.3.3. Adverse events

The overall AE reported were 175. No deaths or serious AE occurred. Almost all (173/175: 98.9%) were mild (2 were moderate, 1.1%), and 172/175 (98%) were recovered (2 of them still not recovered: normocytic-normochromic anemia and salival litiasis; and one unknown: placement of an orthodontic element). The most common adverse events were related to nervous system disorders (81 occurrences presented in 20 participants [83%]). Infections (27 occurrences in 17 participants, 71%) and gastrointestinal disorders (20 occurrences in 13 participants, 54%) were also frequent. Regarding the type of adverse event, the most common was any kind of headache (54 occurrences [30.8%] reported in 16/24 participants [66.7%]). All the participants reported at least one AE, 7 on average. Only two episodes have clinical significance in physical examination during the study. One of them occurred in a placebo (exudative tonsillitis) and the other (salival litiasis) occurred without relation with the vaccination.

Regarding the local AE, one of the most frequent was twitching at the injection site, occurring in all the treatment groups. The intensity of pain in the inoculation point was higher in the groups treated with RUTI® compared to the group treated with placebo, and the pain intensity was also higher when treated with the higher doses. In all the groups treated with RUTI®, the pain intensity was higher after the second inoculation, with the groups 100 and 200 μg of FCMtb being maximal 24 h after each inoculation and almost resolved within 72 h (Fig. 3). The presence of mild–moderate erythema and/or inflammation in the inoculation point was statistically significant different ($p < 0.05$) in groups treated with 100 and 200 μg of FCMtb compared to placebo, 5 and 25 μg of FCMtb groups (Table 4). The presence of induration in the inoculation point was statistically significant different in the group treated with 200 μg of FCMtb compared to the other 4 treatment groups. The most important reaction seen in the inoculation point was swelling in two volunteers, one of them vaccinated with 100 μg of FCMtb and the other with 200 μg of FCMtb. Both lesions were biopsied in order to obtain samples, and both had negative microbiological cultures proving to be sterile and with a pathology result of unspecific granulomatous panniculitis. Both episodes were moderate AE, and were completely recovered in less than 1 month.

The mean AE per participant was higher in groups treated with 5, 100 and 200 μg FCMtb compared to the placebo group. The complete list of systemic and local AE (occurrences vs. subjects) with possible or probably causality to the treatment is summarized in Tables 2–4.

3.3.4. Imunogenicity assays

A wide range of assays to assess the impact of the vaccination on the immune response was planned (Table S2) in order to screen any immunological response enhanced by the vaccination, as well as to determine which assays should be performed in future CTs. A general overview of the results, evaluating the cellular response, showed a dose-related tendency for most of the assays conducted. Highest doses (especially dose of 200 μg of FCMtb) showed the highest results, evidenced in terms of the ELISPOT and ELISA techniques determining the specific IFN-γ secretion. Even though, dose of 25 achieved a polyantigenic response, wider than dose of 100 μg of FCMtb and similar to the one reached by dose of 200 μg of FCMtb, a fact easily recognizable in the ELISPOT assays (Fig. 4). Moreover, even if in the ELISA studies (less sensitive and specific than ELISPOT), doses of 100 and 200 μg of FCMtb showed the highest results, dose of 25 μg of FCMtb evidenced a discrete but clear increase of the IFN-γ secretion attributable to the vaccination (data not shown). Interestingly, the enhanced cellular responses of

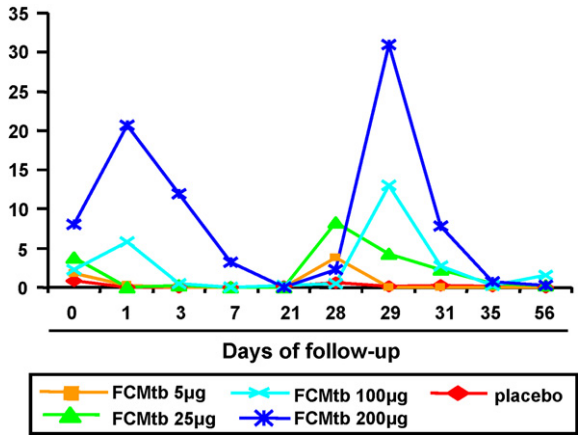

Fig. 3. Subjective pain intensity at the inoculation point (measured with visual analogical scale ranging from 0 to 100) represented for each visit and each treatment group.

Table 2
Systemic adverse events.

Preferred term	Causality	Intensity	Placebo ($n=8$) O/S (%)	RUTI® doses (in μg of FCMtb)			
				5 ($n=4$) O/S (%)	25 ($n=4$) O/S (%)	100 ($n=4$) O/S (%)	200 ($n=4$) O/S (%)
Feeling of body T[a] change	Possibly	Mild	0/0 (0%)	1/1 (25%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
Vasovagal Syncope	Possibly	Mild	0/0 (0%)	0/0 (0%)	2/1 (25%)	0/0 (0%)	0/0 (0%)
Rhinorrea	Possibly	Mild	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/1 (25%)	0/0 (0%)
Sneezing	Possibly	Mild	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/1 (25%)	0/0 (0%)
Lymphadenopathy	Possibly	Mild	1/1 (12.5%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
Nasopharyngitis	Possibly	Mild	1/1 (12.5%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)
Skin rash	Possibly	Mild	2/2 (25%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)

Number (%) of subjects reporting one or more treatment-emergent adverse events and number of AE occurrences by preferred term, possible or probable causality of the treatment and intensity (O = occurrences; S = Subjects).

Table 3
Local adverse events.

Preferred term	Causality	Intensity	Placebo ($n=8$) O/S (%)	RUTI® doses (in μg of FCMtb)			
				5 ($n=4$) O/S (%)	25 ($n=4$) O/S (%)	100 ($n=4$) O/S (%)	200 ($n=4$) O/S (%)
Injection site pain	Possibly	Mild	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/1 (25%)	1/1 (25%)
Twitching	Possibly	Mild	7/4 (50%)	0/0 (0%)	1/1 (25%)	10/4 (100%)	4/3 (75%)
Panniculitis	Probably	Mild	0/0 (0%)	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/1 (25%)
	Possibly	Moderate	0/0 (0%)	0/0 (0%)	0/0 (0%)	1/1 (25%)	0/0 (0%)

Number (%) of subjects reporting one or more treatment-emergent adverse events and number of AE occurrences by preferred term, possible or probable causality of the treatment and intensity (O = occurrences; S = Subjects).

the vaccinated subjects to *M. tuberculosis* antigens could also be detected by the commercial ELISPOT and ELISA assays designed to diagnose latent tuberculosis infection. In terms of the stimuli used to determine the specific IFN-γ mediated cellular response, not all the antigens showed the same evolution after the vaccination. PPD and BCG vaccine were included in both tests because of their nature, as they are both extracts of *M. tuberculosis* and *M. bovis* respectively, and both contain several mycobacterial antigens. That explains the highest results obtained in both assays for these stimuli, and a clearer RUTI® dose–response (Fig. 5). The fact of high background of responses to PPD and BCG could be explained due to the wide incidence of atypical mycobacteria in Spain, a fact that could have been missed for the decision of basing the screening only on the T-SPOT TB result and not the TST result to not influence the Immunogenicity data [28]. For that reason, we do consider that the changes respect to baseline values is more important that the degree of response in itself. Meanwhile, the single antigens were already expected to trigger (if triggered) weaker responses after the vaccination, as RUTI® is an extract of *M. tuberculosis* containing several antigens but all in low concentrations, so the responses obtained were encouraging. The single antigens that showed to trigger the highest responses when used as stimuli for RUTI® groups, were: ESAT-6, 85B, 38 kDa and 16 kDa (more discrete), while antigens 19 kDa and hsp65 only showed responses for one of the assays (ELISA and ELISPOT, respectively). CFP-10 and MPT64 showed weak responses compared to the other antigens tested. This clearly suggests that a polyantigenic response is achieved with the RUTI® vaccination against *M. tuberculosis* antigens secreted by active and latent bacilli, and will help in order to select the more antigenic antigens to test the RUTI® vaccine in future CTs. On the other hand, all the responses obtained tended to be increased after the second inoculation, and then tended to decrease with a later increase at the latest timepoints of the study. The high and maintained responses obtained in the long-term whole blood assay, where the production of IFN-γ secretion by the Memory T-cell subset is evaluated, reinforced this idea (Fig. 6). All this suggests the ability of the vaccination to induce long-lasting protection, even if it should be necessary to follow-up the volunteers in the future to exactly determine how long this protection can last. In terms of % of subsets of T-cells, the RUTI® vaccination enhanced the T-cell populations, as was expected from previous studies conducted in animal models during the pre-clinical development [29]. Interestingly, the results showed that RUTI® achieved an increase attributable to the vaccination especially remarkable for CD4+CD3+ population (doses of 5 and 200 μg of FCMtb) and for CD8+CD3− (doses of 25 and 100 μg of FCMtb). All the RUTI® doses had mycobactericidal activity after incubating the volunteers' whole blood with *M. tuberculosis* (for 72 or 96 h) at least at one timepoint, while the blood of placebo-vaccinated people did not. Even if this ability did not last for long, generally reverting, it is interesting to remark that this could be a useful (even if quite logistically difficult) assay to perform in future CTs, with the mycobactericidal activity a potential surrogate marker of immu-

Table 4
Number of total local adverse events.

Local reaction	Placebo	RUTI® doses (in μg of FCMtb)			
		5 μg FCMtb	25 μg FCMtb	100 μg FCMtb	200 μg FCMtb
Erythema (mild or moderate)	0	0	0	2[†]	3[†]
Inflammation (mild or moderate)	0	0	0	2[†]	5[†]
Induration	0	0	0	0	8[‡]
Abscessification	0	0	0	0	0
Ulcer	0	0	0	0	0
Necrosis	0	0	0	0	0

Statistically significant differences between treatment groups are shown as: (†) meaning significant statistical differences ($p<0.05$) compared to 5, 25 μg and placebo groups; and (‡) as significant statistical differences ($p<0.05$) compared to 5, 25, 100 μg and placebo groups.

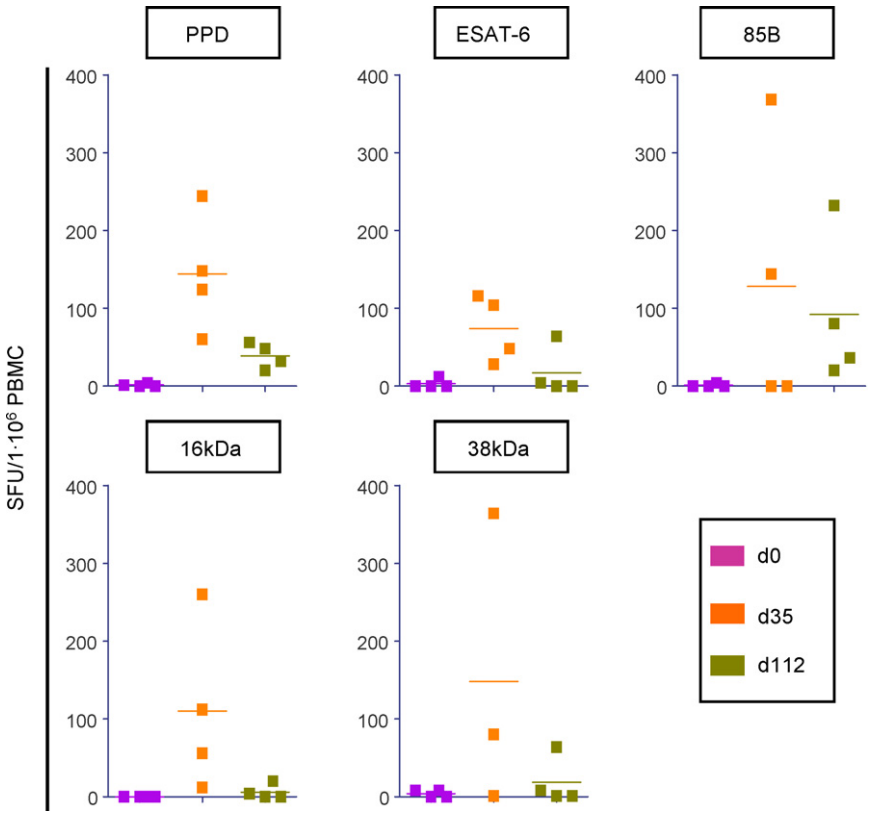

Fig. 4. Cellular immune response (in terms of SFU/1 × 10^6 PBMC) against several *M. tuberculosis* antigens and PPD of volunteers vaccinated with 25 μg of FCMtb. Results of the ELISPOT assay are shown through time, from the baseline value to 1 week after the second inoculation (day 35) and at day 112, almost 5 months after day 0.

nity after RUTI® vaccination of latently *M. tuberculosis* infected people.

The results of the test for the induction of granuloma conducted by the Institut de Pharmacologie et de Biologie Structurale, Toulouse, France, are still being analyzed, thus not being reported in this paper, but we do not exclude publishing them in future.

Humoral immunological response was evaluated by the Pathozyme and the Mycodot assays, as well as with detection of specific antibodies against *M. tuberculosis* recombinant antigens. The Mycodot results were negative for all the volunteers except for 2, one placebo and the other one RUTI®-vaccinated, seeming to be an unspecific technique. The only conclusion that we could extract is that no antibodies against LAM were in general encountered. The Pathozyme assay is a commercial kit ELISA to determine the specific response to 2 of the *M. tuberculosis* antigens, the 16 and 38 kDa ones. A clear increase of the response could be observed after RUTI® vaccination for all doses, even if only the dose of 200 μg of FCMtb reached the positivity marked by the manufacturer's recommendations. The response against *M. tuberculosis* has been classically related to the cellular immunological response, even if the humoral response has been proven to also be a powerful tool to fight *M. tuberculosis* infection as was already shown to be triggered by RUTI® vaccination [30]. Results of the IgM, IgG and IgA antibodies detection assays conducted showed (even if not statistically significantly different) increased levels of IgM and IgG antibodies against *M. tuberculosis* antigens in the RUTI® vaccinated volunteers

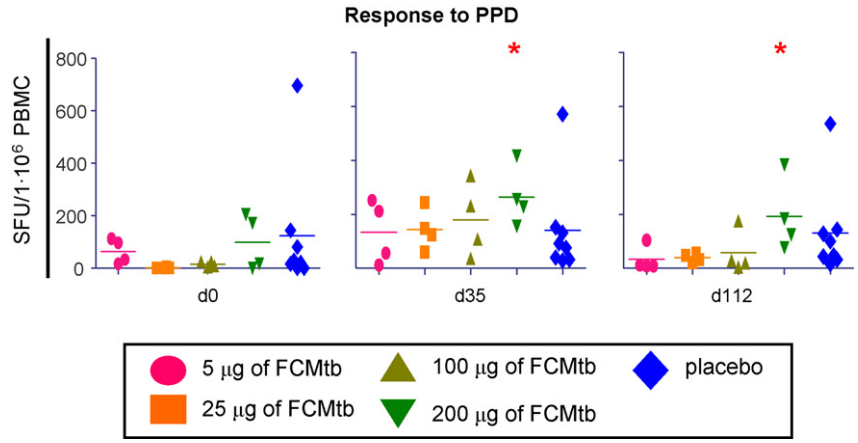

Fig. 5. ELISPOT assay results in response to PPD stimulation of PBMC through time. Each colour represents one treatment. The red asterisk means statistically significant differences compared to placebo ($p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of the article.)

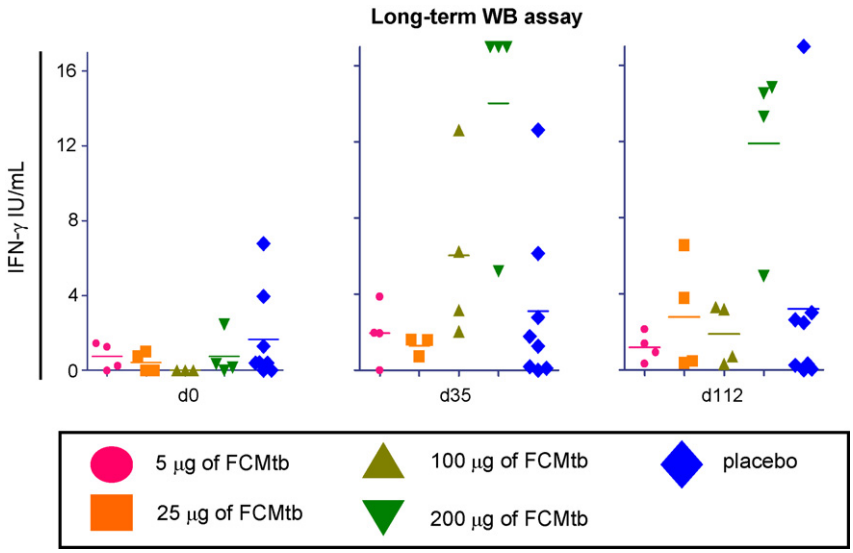

Fig. 6. Long-term whole blood assay results of the volunteers of the 5 treatment groups through time.

when compared to placebo, being clear for the IgM and more discrete for the IgG antibodies. IgG antibodies have been related to disease, while the presence of IgM could correlate to infection with *M. tuberculosis* [31–34]. This idea suggests us that the absence of IgG in our results together with higher levels of IgM antibodies in vaccinated subjects could be directly attributed to RUTI®, without being able to specify if or in which grade they could have a protective role. For all this, it would be very interesting to repeat the assays on these volunteers one year after the vaccination in order to check if this profile has changed, the IgG response being increased or maintained and the IgM response being decreased or cleared. Another reason for mainly finding IgM could also be the need of restimulating the Memory Immunity to produce specific IgG antibodies.

One of the limitations of this study was the amount of techniques conducted, that required a large amount of blood and a very strict schedule at every timepoint and at the end, with some of the assays not providing much useful information. The study to evaluate PCT levels showed a slight increase in RUTI® vaccinees (doses of 25 and 100 μg of FCMtb), but in any case reached the cut-off that limits with the bacterial infection as a cause of inflammation, only suggesting that RUTI® vaccines have an unspecific inflammation with respect to the placebo-vaccinated volunteers. Results from the γδ assay showed no response by any treatment group except for placebo and RUTI® dose of 200 μg of FCMtb at one timepoint, and by RUTI® dose of 25 μg of FCMtb at another timepoint. All the data presented here suggest that it is not worthwhile to perform these two assays in future studies. The granulolysin and the BLT-esterase assays showed increased granulolysin levels (dose of 25 μg of FCMtb) and granzyme activity (doses of 100 and 200 μg of FCMtb) in the RUTI® vaccines, even if they were not high enough to be significantly different from the placebo. These measurements were done because granzymes are molecules related to the inflammatory responses involved in mycobactericidal activity. The results encountered suggest that it would be interesting to test the RUTI® vaccinated subjects for both parameters in future CTs, involving latently *M. tuberculosis* infected population in order to evaluate if the minor differences attributable to the vaccination in these healthy volunteers are major in the infected ones.

Regarding the Immunogenicity assays, Table S4 summarize all results, where statistically significant differences between the groups encountered in the statistical analysis performed are stated. The results of the ELISPOT and of the detected levels of IgM antibodies against *M. tuberculosis* antigens have been added as Figs. 4–6 and Fig. S2, respectively.

4. Discussion

This CT was the first approach to evaluate the safety of the RUTI® vaccine in humans, and the results showed a range of AE in line with other immunogenic products. The incidence of local adverse events was higher in the volunteers vaccinated with RUTI®, and dose-related, the most frequent being twitching in the injection site, with equal frequency in RUTI® and placebo groups, suggesting that this could be related to one of the non-active compounds (excipients) of the final product. The manufacturer of the vaccine is now working on that issue, ameliorating the final product in order to avoid this AE. The more important local reaction seen was swelling with sterile granulomatous panniculitis in 2 out of 24 volunteers. Even if its incidence was low (2/16), both being moderate and fully recovered, by their nature and occurrence with the highest doses, these AE could recall the Koch reaction obtained after the therapeutical use of tuberculin in tuberculosis, a fact related to the dose and the dosage timing used, as stated in the several classical studies of the beginning of the 20th century [35,36]. One could fear the incidence of this AE to be higher in infected, i.e. immunized people and to appear after the first dose even if using lower doses. The safety results are in line with those obtained in previous studies carried with similar products, even if it is difficult to compare results when there are differences in vaccines' origin, different regimens and target population. Inflammation, swelling and other local reactions have been recorded in other CT involving healthy people [16], and are very common in CT evaluating boosting vaccines [12,37,38]. We do believe the AE due to RUTI® in future trials conducted in LTBI populations could be similar to those encountered in trials involving vaccines in prime-boost regimens. In this regard, the experiences with tuberculin showed that the dosage range is crucial to achieve an improvement of the immunological response without impairing the safety [36,39]. All these aspects are currently evaluated when deciding the doses to test in further studies, designing them with precision to avoid any toxicity that could harm future volunteers, the results of the present study being very useful to establish the appropriate dosage range.

This CT also constitutes the first approach to study the mechanisms of action of the RUTI® vaccine in humans. The results obtained shown us that a cellular and humoral specific immunolog-

ical response was triggered by the vaccination, which sometimes were statistically significantly different from the one obtained in the volunteers vaccinated with placebo, and even when not, a tendency to increased responses was noteworthy. The variability of responses to the vaccination among the individuals included in the trial was random, and even if the sample size was selected according to any other Phase I CT of this kind, we do believe that a higher sample size could have obtained more statistically significant differences between the treatment groups in spite of this intrinsically individual variability to the interventions. Another pitfall when interpreting the results was also the high background that sometimes was detected for some of the treatments: specific responses against some of the antigens obtained at day 0, or for the placebo group at some timepoints. Those results being due to some of the individuals that constituted each group, and even without interfering in the global analysis which demonstrate the superiority of RUTI® compared to placebo in terms of immunological responses, a deep analysis of each volunteer condition is being conducted, in order to better understand the results obtained. Consequently a new study is being planned in order to follow all these volunteers and their evolution. As explained before, we believe that having a bigger population for the CT could have minimized these interindividual differences, homogenizing the whole population for each treatment and thus helping to better show the real boosting effect that occurs after RUTI® immunisation.

None of the volunteers included were infected with *M. tuberculosis* nor BCG vaccinated (thus this being their first time facing bacilli's antigens), a fact that will not be repeated in further studies, where the target population will be previously infected people. For this CT being conducted in totally healthy volunteers, the immunological responses obtained can be considered noteworthy, and in line with CT testing other vaccines in healthy people [12,16], even if comparisons are difficult due to different designs and outcomes. Higher responses are expected in the already *M. tuberculosis* infected individuals, as they will be already primed.

None of the current vaccines against *M. tuberculosis* infection based on *M. tuberculosis* presently under clinical development are tested as therapeutical agents, thus no real comparison could be made with the results obtained in this CT.

Until the present, it can be concluded that the safety of the vaccine has been demonstrated in healthy volunteers, and RUTI® has also shown to trigger a specific response against *M. tuberculosis*, ensuring the clinical development through further studies.

Acknowledgments

Participating investigators: Nuria López, Sandra Anzola, Eva Portabella and Francisca García worked collecting data and provided and cared for study patients. Jordi Lara and Sandra Serrano worked in the statistical analysis of all the data collected during the trial.

Other contributions: We would like to give special thanks to the 24 participants of this CT, as well as all the volunteers willing to participate that could not finally be included. We also would like to thank the Pneumology, Dermatology, Pathology and Microbiology Departments of the Hospital Germans Trias i Pujol, where this trial was conducted, for helping in the follow-up and diagnosis of the AE, especially Dr. J. Ruiz Manzano, Dr. J. Ferrándiz, Dr. Tapia and Dr. C. Prat.

Innate Pharma (Marseille, France) kindly provided the BrHPP to run the γδ Tcells assay.

The Institut de Pharmacologie et de Biologie Structurale (Centre National de la Reserche Scientifique, Université Paris Descartes, Toulouse, France) tested the whole blood samples for the induction of granuloma.

The homemade ELISA assays to test sera samples for antibodies against *M. tuberculosis* recombinant antigens were carried out by Lionex Diagnostics and Therapeutics GmbH (Braunschweig, Germany), thus Mahavir Singh, Ralf Spallek and Susanne Kämpfer.

Dr. F. Dieli from the Dipartimento di Biopatologia e Metodologie Biomediche Università di Palermo (Italia) tested the sera samples for BLT-esterase and granulolysin.

Competing interests: PJC is co-inventor of the patent claiming of RUTI® as therapeutic vaccine. Regulatory approval and further clinical development is being undertaken by a spin-out the biopharmaceutical company Archivel Farma, s.l. in collaboration with the Institut Germans Trias i Pujol. PJC is the Scientific Director of this development.

Funding: As the owner and manufacturer of the vaccine to be studied (RUTI®), Archivel Farma s.l. (Badalona, Catalonia, Spain) was the sponsor of this trial and funded it. The funders had no role in data collection and analysis or the preparation of the manuscript. As stated in the protocol of the trial, the investigators and the sponsor agreed on to disclosure of the results obtained.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2009.09.134.

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