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MTBVAC, a live TB vaccine poised to initiate efficacy trials 100 years after BCG

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ABSTRACT

At its 100th birthday of its first administration to a newborn, BCG has been (and continues being) an inspiration for the construction and development of hundreds of new TB vaccine candidates in the last two and a half decades. Today, 14 candidates are in clinical development inside the global TB vaccine pipeline. MTBVAC is one of these candidates. Based on a live-attenuated *Mycobacterium tuberculosis* clinical isolate, MTBVAC's 25 years of vaccine discovery, construction and characterisation have followed Pasteur principles, and in the process, BCG has served as a reference gold standard for establishing the safety and protective efficacy of new TB vaccine candidates. MTBVAC, which contains the antigen repertoire of *M. tuberculosis*, is now poised to initiate Phase 3 efficacy trials in newborns in TB-endemic countries. BCG's efficacy extends beyond that against TB, shown to confer heterologous non-specific immunity to other diseases and reduce all-cause mortality in the first months of life. Today, WHO recognises the importance that any new TB vaccine designed for administration at birth, should show similar non-specific benefits as BCG *via* mechanisms of trained immunity and/or cross-reactivity of adaptive immune responses to other pathogens. Key recent studies provide strong support for MTBVAC's ability of inducing trained immunity and conferring non-specific heterologous protection similar to BCG. Research on alternative delivery routes of MTBVAC, such as a clinically feasible aerosol route, could facilitate vaccine administration for long-term TB eradication programmes in the future.

1. Global tuberculosis epidemiology and the need for new vaccines

The Bacille Calmette-Guérin (BCG) centenary vaccine is one of the most widely used of all current vaccines in the world [1,2]. It is on the World Health Organization's (WHO's) list of essential medicines [3] and since 2004, globally about 100 million children are vaccinated with BCG each year [4]. In infants, in addition to decreasing all-cause mortality, BCG confers protection (although not absolute) against the disseminated forms of the disease, miliary and meningeal tuberculosis (TB) [4]. However, in adolescents and adults, BCG's efficacy is more variable, ranging from 0 to 80% in different parts of the world, depending on factors including socioeconomic conditions, HIV-status, presence of environmental mycobacteria, or climate and air polution, among others [5]. Another possible hypothesis for BCG's variable efficacy in adults and adolescents is that its protective immunity is thought to last for up to 10–15 years [1,4,5], although there is evidence for longer protection [6–8].

Despite BCG's widespread use, TB remains the leading representative respiratory tract communicable disease threatening public health, demonstrating the need to improve the efficacy of BCG in protecting against respiratory TB, the transmissible form of the disease. And although incidence and mortality rates have slowly been declining since the turn of 21st century, there were an estimated 10.0 million new cases and more than 1.4 million deaths reported on a global level in 2019 by WHO [1]. TB mortality has only been surpassed by the COVID-19 pandemic which in 2020 caused 1.8 million deaths as reported to WHO [9]. TB and COVID-19 are both respiratory transmission diseases and it is estimated that as result of lockdown-related disruptions due to COVID-19 pandemic, deaths from TB could increase by up to 16% in the next five years [10]. In high-income countries, TB (like COVID-19) mainly affects the elderly, whereas in low resource settings, each year TB primarly takes the lives of young and middle-aged adults, the most productive members of society.

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Globally, some 50 million individuals are already latently infected with multi- and extensively-drug resistant (MDR and XDR, respectively) *M. tuberculosis* strains creating a remarkable resource for future cases of active TB with insufficient treatment options. Nevertheless, the WHO End TB Strategy has vowed to reduce TB morbidity by 90% and TB mortality by 95% by 2035 and recognizes the urgent need for more accessible diagnostic tools that are rapid and reliable, new less toxic and more efficacious antibiotics to shorten therapy and ultimately new vaccines to prevent pulmonary TB in order to achieve this ambitious goal [11].

2. Current TB vaccine pipeline

Based on a high unmet medical need, the development of new TB vaccines has been identified as a priority for WHO Initiative for Vaccine Research and in 2018, WHO developed a Preferred Product Characteristics (PPC) document for new TB vaccines with the aim to cover the priority need for vaccines that protect against pulmonary TB in adults, and new TB vaccines with better safety and efficacy characteristics than BCG to administer to neonates and infants [12]. The PPC for new TB vaccines includes WHO's preferences for parameters, such as vaccine indications, target groups, possible immunization strategies, and features of desired clinical data related to safety and efficacy, supportive of policy decision making [12].

The last two and a half decades of intensive research, have led to a number of new TB vaccine candidates in clinical development. Today, the global TB vaccine pipeline features 14 vaccine candidates in different stages of clinical development, from Phase 1 through Phase 3 [13,14]. These candidates fall into one of three different concepts: preventive pre- and post-exposure subunit vaccines based on one or more recombinant *M. tuberculosis* fusion proteins, either vehiculated by viral-vectored or administered with adjuvant, whose goal is to boost immunity to BCG; killed or fractioned whole cell vaccines, for therapeutic purposes; and live-attenuated mycobacterial vaccines, which target either BCG-replacement at birth and/or prevention of TB in adolescents and adults. The greatest challenge in the development of vaccines against TB is understanding the mechanisms by which *M. tuberculosis* evades and escapes the immune responses of the host. Without knowing the immunological bases of protection against TB, it is very difficult to design vaccines with a limited number of antigens that can protect against the disease. As Professor Douglas Young emphasizes, we need new vaccines against TB that protect against the disease and we need to understand the immunology of the disease in order to obtain these vaccines [15]. That is why it is very important to study the host-pathogen immune dinamycs of *M. tuberculosis*, as 90% of those infected with TB do not develop the illness.

The advancements in mycobacterial genetics were first made in the 1980 s, with more than 20 years of delay with respect to *Escherichia coli*, mainly due to the fastidious manipulation and the pathogenicity of some mycobacterial species. The delay in developing mycobacterial genetic tools was reflected in the construction of new live attenuated vaccines against TB. Professor Brigitte Gicquel pioneered the development of mycobacterial genetics through techniques that allowed to introduce DNA into mycobacteria as well as develop gene inactivation protocols in *M. tuberculosis* [16–21]. These methodologies allowed to construct MTBVAC from a clinical isolate of human origin. The construction of such live attenuated vaccines is tedious and a more laborious strategy compared to other vaccine candidates, since it involves starting from scratch and having to demonstrate in a first stage the attenuation and safety of the candidate vaccine and then the increased immunity to TB. In 2005, a consultation meeting was held in Geneva where scientists, stakeholders, vaccine developers and manufactures, and regulators met with the aim of defining the safety criteria for live-attenuated mycobacterial vaccines to reach first-in-human Phase 1 clinical evaluation, and as a result, a consensus statement was developed [22] (referred to as the Geneva consensus throughout the text). MTBVAC was designed and constructed fulfilling these same Geneva consensus requirements for entry into first-in-human clinical trials, namely, containing two stable deletion mutations without antibiotic resistance markers to avoid reversion to virulence, and demonstrating in recognized TB-relevant animal models similar safety and bio-distribution profiles as BCG [23] and improved protection against TB [24–27]. Today, MTBVAC remains the first live attenuated vaccine, genetically modified, based on the human pathogen *M. tuberculosis* fulfilling the Geneva consensus requirements in ongoing successful clinical developmpent.

In agreement with regulatory, WHO and the Geneva safety requirements for development of live mycobacterial vaccines, BCG has served as the reference gold-standard comparator in the preclinical, clinical and industrial development of MTBVAC. Thanks to BCG, today MTBVAC is the first vaccine of its kind that has successfully reached clinical evaluation and is now poised to initiate Phase 3 efficacy trials in newborns in South Africa in 2022. Following the WHO PPC document, if MTBVAC is able to demonstrate improved safety and/or significantly greater efficacy than BCG when administered in newborns in high-burden TB-endemic settings, MTBVAC could eventually replace BCG.

The main advantage of using live vaccines based on rational attenuation of *M. tuberculosis* is their ability to keep the genetic repertoire encoding immunodominant antigens absent in BCG, whereas chromosomal deletions in virulence genes provide assurance for safety and genetic stability. Such vaccines are expected to safely induce more specific and longer lasting immune responses in humans that can provide protection against all forms of the disease. This is the rationale that has been followed in the development of the live-attenuated MTBVAC.

3. MTBVAC conceived following BCG's rationale and Pasteur's principles for the development of vaccines

MTBVAC and BCG are both live-attenuated derivatives of the TB pathogens in humans (*M. tuberculosis*) and in bovids (*Mycobacterium bovis*), respectively. Both *M. tuberculosis* and *M. bovis* are related members of the *M. tuberculosis* complex (MTBC), characterized by more than 99.95% similarity at the nucleotide level, identical 16S rRNA sequences and a strictly clonal population structure without "recent" evidence of ongoing horizontal gene transfer between them [28]. Both *M. tuberculosis* and *M. bovis* emerged after an evolutionary bottleneck from a most recent common ancestor [29]. Today, global genome sequencing analyses have shown that relative to the human-adapted pathogen *M. tuberculosis, M. bovis* has smaller chromosomal content due to numerous genomic deletion events, namely lacking various regions of difference (RDs), ranging from RD4 to RD11 [30]. Specifically, loss of RD9 and other specific RDs in *M. bovis* allow to branch it off from *M. tuberculosis* in the evolutionary tree of the MTBC.

As result of the repeated subcultivation process of *M. bovis* BCG to achieve its attenuation between 1908 and 1921, BCG contains about 100 genes less relative to *M. tuberculosis* [29]. Among them, deletion of RD1, which encodes the protein secretion system ESX-1, is considered the most relevant deletion responsible for BCG attenuation [30]. It is generally accepted that T-cell epitopes are evolutionarily hyper-conserved in the MTBC [31,32] and as such, it is important to remark that regions lost in BCG strains also

contain potent antigenic proteins. After an updated analysis based on previous studies [33,34], we found that of the 1,603 experimentally validated human T cell epitopes in *M. tuberculosis*, 433 (27%) of them are located in RD regions absent in BCG Pasteur. These epitopes are distributed in RD1 (307 epitopes), RD2 (59 epitopes), RD11 (42 epitopes), and small percentages in RDs 3–10, 13 and 14. What is more, the vast number of epitopes absent in BCG is located in only five antigenic proteins within the RD1, namely ESAT6 (112 epitopes), CFP10 (95 epitopes), and PPE68 (79 epitopes) and within RD2, namely MPT64 (24 epitopes) and Rv1985c (23 epitopes) [35]. These studies further support the hypothesis that epitope sequence variation in BCG potentially affects human T cell recognition that could result in ineffective priming of the host immune system [33,34,36,37].

4. MTBVAC design following Pasteur's principles: Isolation of the human pathogen, attenuation by inactivation of selected genes, protection in animals, and evaluation in humans

Which genes to select for attenuation of *M. tuberculosis*? BCG was conceived as an attenuated strain of *M. bovis* that caused TB in cows and was transmitted to humans mainly through the ingestion of unpasteurized milk. In a similar manner, the conception of MTBVAC stems from what we learned from the deadly outbreak in Spain in the early 1990's caused by an epidemic strain of multidrug-resistant (MDR) *M. bovis* responsible for more than 100 deaths among HIV-patients, before retroviral treatment was available [38–40]. Given that it is highly unusual for *M. bovis* to be transmitted among humans, we performed a molecular characterization of this unusual *M. bovis* strain, which today is well characterized as extensively drug-resistant (XDR) [41], and we discovered that an unexpected IS*6110* insertion upstream the *phoP* gene led to increased transcription of this gene and accounted for the increased transmission and virulence of *M. bovis* [38,42,43] (Fig. 1**A**). This led us to hypothesize that PhoP, which is part of the two-component system PhoP/PhoR, could be essential for the virulence of *M. tuberculosis* and its inactivation in a clinical strain would attenuate the virulence of the human tubercle bacillus [43] (Fig. 1**B**).

MTBVAC is a live rationally attenuated *M. tuberculosis* derivative of Mt103 strain, a clinical isolate from a TB patient, that we chose instead of a laboratory *M. tuberculosis* strain, such as H37Rv, Erdman or CDC1551, to avoid undesired laboratory adaptations as result of successive subcultivation *in vitro* [23]. Mt103 is a modern *M. tuberculosis* strain belonging to Lineage 4, which, together with Lineage 2 (Beijing strains), represents the most geographically widespread lineage of the MTBC transmitted by the aerosol route between humans [44]. MTBVAC was constructed and characterised fulfilling the Geneva consensus safety requirements for entry into clinical evaluation [22], and in this regard it was designed to contain two independent stable genetic deletions, without antibiotic resistance markers, in the genes *phoP* (Rv0757) and *fadD26* (Rv2930) encoding two major virulence factors [23]. The gene *phoP* was chosen because of our previous observations that the two-component system PhoPR is essential for *M. tuberculosis* virulence [43]. PhoP has been shown to regulate more than 2% of *M. tuberculosis* genes, most of which are implicated in virulence [45], and this regulation could be mediated through other transcription factors, e.g., WhiB6 of the ESX-1 system [46] or *via* non-coding RNAs, like the described *mcr7* and its role in regulating the TAT secretion system, leading to over secretion of major antigens, such as those of the Ag85 complex in *M. tuberculosis phoP* mutants, as MTBVAC [47]. Other relevant virulence genes regulated by PhoP include lipid metabolism genes (e.g., *pks2, pks3*) involved in biosynthesis of the polyketide-derived acyltrehaloses (DAT, PAT) and sulfolipids, which are front-line lipid constituents of the cell wall thought to have a role in host immune modulation, interfering with the recognition of *M. tuberculosis* by the immune system [48,49]. PhoP also regulates genes within ESX-1 (e.g., the *espACD* locus) implicated in the secretion of the major antigen and virulence factor ESAT6, so that *phoP*-mutants can produce but are

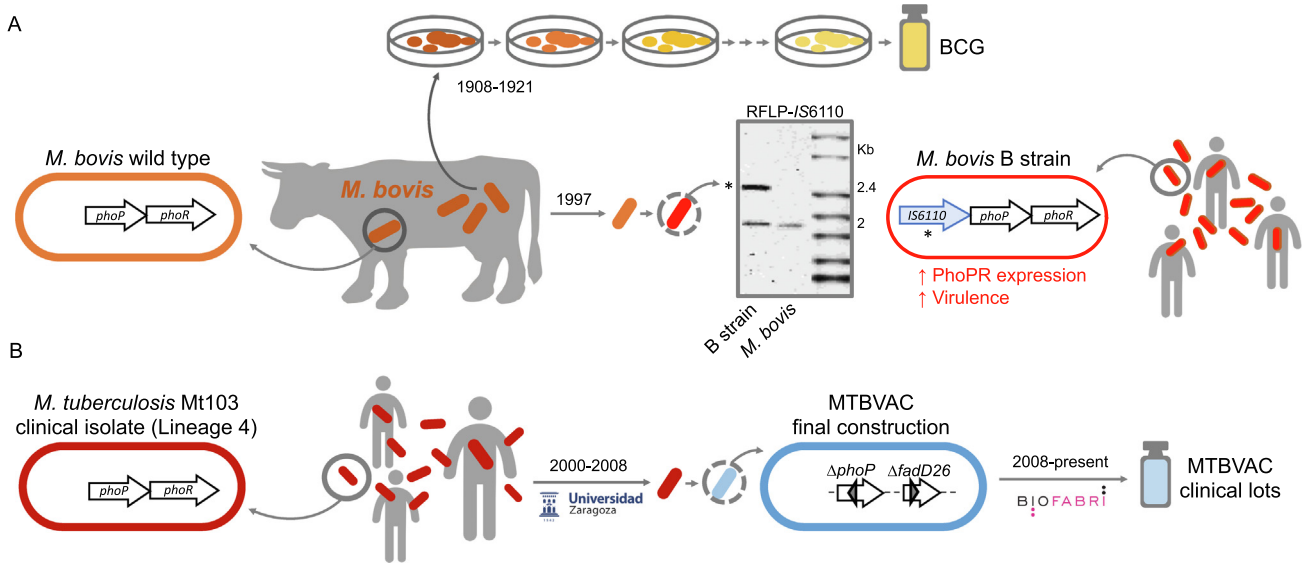

Fig. 1. Discovery the essential role of *phoP* in *M. tuberculosis* virulence and construction of MTBVAC. (**A**) In 1908, Calmette and Guerín started repeated subcultivation passaging of a virulent bovine strain *M. bovis*, supplied by Nocard to them (originally isolated by him in 1902 from the udder of a tuberculous cow) and by1913, Calmette and Guerín had achieved an attenuated version of the *M. bovis* strain, which they named BCG, and started testing it in animals before its first administration to a newborn in 1921 [78]. In 1997, we published for the first time the molecular characterization of the *M. bovis* strain responsable for a deadly outbreak among HIV-patients in Spain [41], containing an IS*6110* insertion upstream the *phoP* gene leading to its increased transcription. (**B**) Inactivation of *phoP* and demonstration of its essential role in virulence of *M. tuberculosis*, as first step toward the construction of MTBVAC. In 2008, MTBVAC final vaccine construct was ready to start GMP development, fulfilling Geneva consensus requirements for entry into first-in-human clinical trials, containing two stable genetic mutations without antibiotic resistance markers in the virulence genes *phoP* and *fadD26*.

unable to export ESAT6 [22,50,51]. Besides, in order to fulfil the Geneva requirements, a second gene implicated in virulence was inactivated in MTBVAC. The *fadD26 gene* is the first gene in an operon required for the biosynthesis and export of phthiocerol dimycocerosates (PDIM), the main virulence-associated cell-wall lipids of *M. tuberculosis* [52–55]. Recent evidence indicates that PDIM are involved in phagosomal rupture in concert with ESAT-6 [52,56,57].

5. Attenuation and protection in different animal models: mice, guinea pigs, non-human primates

As outlined in the second Geneva consenus requirements for live-mycobacterial vaccines, all preclinical characterization studies of new TB vaccine candidates should use BCG as reference gold-standard comparator, so that any new live attenuated vaccine should be at least as safe as BCG and show significant improvement of TB disease in the relevant animal models, mouse, guinea-pigs, and non-human primates [51] (Fig. 2). Between 2001 and 2011, rigorous preclinical safety, protective efficacy and immunogenicity studies were conducted with MTBVAC in recognized TB-relevant animal models by independent (national and international) laboratories inside research projects funded by the European Union through consecutive Research Programs [23,27] (Fig. 2).

The preclinical studies conducted in small animal models (mice and guinea pigs) showed that MTBVAC vaccination is safe, immunogenic and has the potential to enhance protection against experimental *M. tuberculosis* challenge relative to BCG [23,27]. Briefly, MTBVAC demonstrated to be at least as safe as BCG in immunocompromised SCID mice, and to confer greater or equivalent immunogenicity and protective efficacy to BCG against experimental pulmonary *M. tuberculosis* challenge in mice and guinea pigs [23,25,58,59]. In more recent studies in adult and newborn mouse models, MTBVAC was shown to confer improved protection compared to BCG [24,58]. All these studies provided strong bases for the progress of MTBVAC to first-in-human clinical evaluation in adults and newborns. To that end MTBVAC was developed by the Spanish Biopharmaceutical company Biofabri as a freeze-dried formulation in compliance with current Good Manufacturing Practices (GMP) and fulfilling regulatory guidelines for assuring the quality, safety, and efficacy of BCG freeze-dried vaccine [23] (Fig. 2).

6. From the lab bench to regulatory approval to enter first-in-human clinical evaluation

For receiving regulatory approval to enter first-in-human Phase 1a clinical evaluation of MTBVAC in healthy adults in Lausanne,

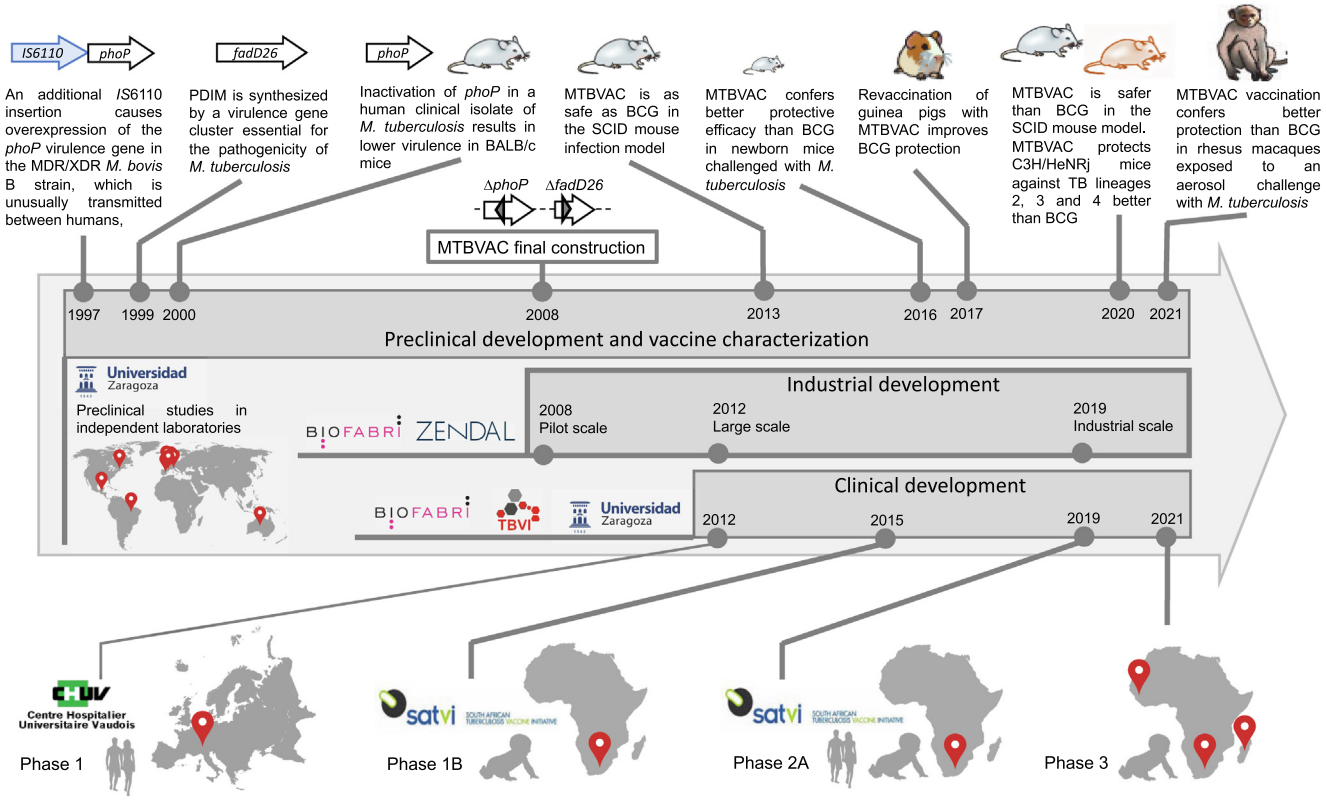

Fig. 2. MTBVAC from discovery to human clinical evaluation abiding by Pasteur's principles on vaccine development. Identification of virulence genes *phoP* and of PDIM locus in *M. tuberculosis* (1997–2001) settled the basis for MTBVAC construction (2005–2008). 2001 until present: ongoing preclinical characaterization studies deciphering mechanisms of vaccine safety and induced protection in different recognized TB-relevant animal models conducted by different national and international laboratories: mouse studies (Unizar and other laboratories in Spain), guinea pigs [Public Health England (Ann Williams and Simon Clark)] and non-human primates [Public Health England (Sally Sharpe) and Bimedical Primate Research Center (Frank Verreck, the Netherlands)]. 2008–2012: GMP development of freeze-dried MTBVAC. 2012 until present: Industrial development and scale-up production of MTBVAC. 2012: First-in-human Phase 1a clinical evaluation of MTBVAC in healthy adults in Lausanne, Switzerland (funded by TBVI with funding from Bill & Melinda Gates Foundation). 2015: successful Phase 1a completion leads to first-in-human Phase 1b evaluation of MTBVAC in newborns in South Africa in collaboration with the South African Tuberculosis Vaccine Initiative (SATVI) (NORAD funding). 2019: MTBVAC enters two dose-defining Phase 2 trials in South Africa, one in adults (in collaboration with IAVI, US) and another one in newborns (EDCTP2-funded, see acknowledgements), which includes capacity building studies in Senegal and Madagascar. 2021: MTBVAC received EDCTP funding for a Phase 3 efficacy evaluation in newborns in South Africa, Senegal and Madagascar. Biofabri is the exclusive licensee of MTBVAC and industrial developer and Sponsor of MTBVAC clinical trials. Unizar is MTBVAC intellectual rights owner and vaccine discoverer, and TBVI is the European Tuberculosis Vaccine Inititiative. Since 2008, Biofabri works in close collaboration with Unizar and TBVI in the GMP and clinical development of MTBVAC.

Switzerland, scientific advice was sought from National (country of manufacture (Spain)) and Swiss regulatory authorities (country of the first-in-human Phase 1a clinical trial). After thorough review of all preclinical data, additional biodistribution and formal toxicology studies were designed and executed according to regulators' advice to achieve an in-depth characterization of the final freeze-dried vaccine formulation. The studies incorporated formal toxicity and local tolerance in mice, and comprehensive up-to 6-month organ distribution, persistence/clearance, and excretion studies in well-established mouse and guinea-pig models of TB. All studies employed freeze-dried clinical grade BCG SSI as the reference gold standard comparator, same strain used as control in MTBVAC Phase 1a trial. Moreover, the *in vitro* and in vivo (in guinea pigs) GMP characterization assays for lot release of freeze-dried MTBVAC were established following Euroepan Pharmacopoeia and WHO guidelines for freeze-dried BCG production and using freeze-dried clinical grade BCG SSI as the reference gold-standard comparator. In summary, the additional safety and toxicity studies showed that MTBVAC freeze-dried formulation is at least as safe as BCG SSI and appears to be less reactogenic in mice and guinea pigs [23]. Studies in newborn mice showed that freeze-dried MTBVAC and BCG SSI present comparable safety and lack of toxicity profiles, as measured by health appearance, normal growth and development behaviour, and survival until fixed endpoint (6 months) of experiment, and no effects on organ development at necropsy [58].

The success of the preclinical and GMP development of MTBVAC to support first-in-human Phase 1 clinical evaluation was the result of the close collaborative partnership between the vaccine developers Biofabri, University of Zaragoza (Unizar), and the European not-for-profit TuBerculosis Vaccine Initiative (TBVI) [13]. Since 2008, the MTBVAC project has received external experts' advice on preclinical, regulatory, GMP and clinical development facilitated through the TBVI Product and Clinical Development Teams (TBVI PDT and CDT), acknowledged in [23,27] (Fig. 2). All preclinical data combined with the availability of GMP produced and characterised freeze-dried MTBVAC clinical lots led to successfully obtaining regulatory approval by Swiss Medical Regulators (Swissmedic) to enter first-in-human clinical evaluation in healthy adults in Lausanne, Switzerland in Oct 2012 [23,27,60] (Fig. 2).

7. MTBVAC poised to initiate Phase 3 efficacy trials in TB-endemic countries of Sub-Saharan Africa

After 11 years of intensive research and animal studies, the approval in Oct 2012 to enter first-in-human dose-escalation Phase 1a clinical evaluation of MTBVAC was an important milestone. Conducted at Lausanne University Hospital (CHUV) in Switzerland by the study principal investigador (PI) Prof. François Spertini, the study's primary objectives were safety and local tolerance of MTBVAC in comparison with BCG SSI with minimum secondary immunogenicity in healthy adult volunteers without any history of BCG vaccination, or exposure to TB (PPD-negative) and or HIV [60]. Successful completion of the trial demonstrated that intradermal vaccination with MTBVAC was at least as safe as BCG and did not induce serious adverse events. This Phase 1a trial marked a historic milestone in human vaccinology as for the first time a live-attenuated *M. tuberculosis* vaccine was successluy tested in humans corroborating its safety and immunogenic potentional, supporting clinical development in high-burden TB-endemic countries.

Today the Spanish biopharmaceutical and vaccine manufacturer Biofabri is the exclusive licensee and industrial and clinical developer of MTBVAC, also Sponsor of the clinical trials. In partnership with TBVI and MTBVAC intellectual owner University of Zaragoza, Biofabri has established two independent clinical development plans (CDPs) for intradermal MTBVAC. The primary CDP focuses on intradermal MTBVAC as a preventive vaccine administered in newborns (BCG replacement strategy). The secondary CDP, also in collaboration with US International AIDS Vaccine Inititiative (IAVI) (continuation of the former US-founded Aeras), aims at the development of MTBVAC as a preventive vaccine in BCG-vaccinated adolescents and adults living in high-burden countries with or without prior infection with *M. tuberculosis*.

Following the first-in-human Phase 1a trial, MTBVAC was evaluated in a dose-escalation Phase 1b safety and immunogenicity trial in South Africa in its primary target age group population, namely healthy HIV-unexposed newborn babies [61] (Fig. 2). Conducted by the South African Tuberculosis Vaccine Initiative (SATVI) with PI Dr. Michele Tameris, the Phase 1b trial included a safety arm in healthy adults as a safety step prior to vaccination of newborn babies. MTBVAC Phase 1b trial confirmed vaccine safety and a greater immunogenicity as compared to the same dose of BCG SSI, suggestive of the broader antigenic content.

MTBVAC is now completing two dose-defining Phase 2 trials in South Africa at SATVI, using BCG as reference comparator. A Phase 2a in newborns (NCT03536117) and a Phase 1b/2a in BCG-vaccinated adolescents and adults with and without prior *M. tuberculosis* infection (NCT02933281). The vaccination phase has been completed and safety and immunogenicity results will allow the dose-definition of MTBVAC for entry into already planned multicenter Phase 3 effiacy evaluation in newborns in Sub-Saharan Africa. MTBVAC project was recently awarded partial funding through The European & Developing Countries Clinical Trials Partnership (EDCTP2) to conduct a multi-center Phase 3 efficacy trial in newborn babies in South Africa, Madagascar and Senegal, which is now estimated to initiate second quarter of 2022 to account for delays in the Phase 2 trials caused by the COVID-19 pandemic in 2020 (Fig. 2).

8. Key recent efficacy data in non-human primates supporting Phase 3 efficacy evaluation

Since the early stages of clinical development of MTBVAC, National and European Regulators have highlighted the importance of evaluating the immunogenicity and efficacy of freeze-dried MTBVAC in a superior animal model such as non-human primates (NHP) in order to provide data to accelerate and assist decisions to move to efficacy trials. In this respect, multi-faceted efficacy studies of MTBVAC in a macaque *M. tuberculosis* model, promoted by former Aeras (with funding from Bill and Melinda Gates Foundation), were conducted at Public Health England. Part of the study was recently published by White *et al.* by the group of Sally Sharpe [26], demonstrating that a single intradermal vaccination with MTBVAC given to BCG-naïve adult rhesus macaques was well tolerated and conferred a significant improvement in protective efficacy outcome following aerosol exposure to *M. tuberculosis* compared to that provided by a single BCG vaccination. White, A. *et al.,* also describe concordance between immune profiles measured in the MTBVAC clinical trials and a significantly improved outcome after *M. tuberculosis* challenge as evidence to support the continued development of MTBVAC as an effective prophylactic vaccine for TB vaccination campaigns.

Relevant to the secondary CDP of MTBVAC, aiming at application of MTBVAC in BCG-vaccinated adolescents and adults in TB-endemic countries, where BCG is usually given at birth, revaccination studies in guinea pigs showed superior protection when intradermal MTBVAC was given as boost following BCG vaccination as compared to BCG alone [25], indicating that, rather than boosting the waning efficacy of BCG, a vaccination schedule involving a

combination of these two live vaccines could be able to yield stronger immunity to *M. tuberculosis* infection.

9. In the footsteps of BCG: MTBVAC in diverse therapeutic settings

As it has been widely explored in the last decade and a half, continuous evidence is emerging demonstrating that apart from providing specific protection against *M. tuberculosis* infections, BCG vaccination confers significant reduction of non-tuberculous infectious diseases and all-cause mortality in the first 6 weeks of life in infants [62]. This non-specific protection is thought to be related to cross-reactivity of the adaptive immune system with unrelated pathogens [63], and to training of the innate immune system by means of metabolic and epigenetic reprogramming of innate immune cells through a process termed "trained immunity" [62,64]. Thus, prioritisation of BCG on the first day of life in high-mortality settings is suggested to have significant public-health benefits through reductions in all-cause infectious morbidity and mortality [62]. In this regard, the WHO PPC document for new TB vaccines considers the importance of establishing whether any new TB vaccine candidates (especially those designed as BCG-replacement strategy), currently under investigation, are also able to induce trained immunity and initiate similar heterologous protective effects [12].

In the context of MTBVAC, its ability to be applied in different therapeutic settings has been increasingly exploited by our group. The potential of MTBVAC as a bladder cancer therapy *in vitro* and in vivo in a preclinical model was first published three years ago, demonstrating MTBVAC's ability to colonize human bladder tumor cells to a much greater extent than BCG and a higher antitumor effect in a murine model in vivo [65]. Intravesical instillation of BCG has also been used as a first-line therapy for non–muscle-invasive bladder cancer. Very recenlty, we showed the capacity of intranasal administration of BCG or MTBVAC to revert established asthma in mice, suggesting that these live vaccines might be triggering allergen-specific memory T cells with a Th1 profile [66]. Also in a collaboration with the group of Prof. Mihai Netea [67], we showed that MTBVAC is able to generate trained immunity *in vitro* in human primary myeloid cells, which resulted in an enhanced response after secondary challenge with non-related bacterial stimuli. Importantly, these findings were complemented with *in vivo* studies in mice demonstrating ability of MTBVAC to induce trained immunity in immunocompetent and immunocompromised SCID mice and to confer heterologous protection against a lethal challenge with *Streptococcus pneumoniae* in an experimental murine model of pneumonia. All these data underline the potential of MTBVAC as a candidate for universal vaccination against TB or treatment against bladder cancer, representing a potential alternative to the current BCG vaccines . Most recently, we also found that when administered before DTaP in mice, both BCG and MTBVAC were able to trigger effective Th1 immune responses against diphtheria, tetanus, and pertussis and enhanced humoral responses against DTaP antigens. Moreover, exploration of human epidemiological data showed that pertussis incidence was 10-fold lower in countries that use DTaP and BCG compared to countries that use only DTaP suggesting potential beneficial impact of BCG vaccination on the protection against pertussis conferred by DTaP [68] (Table 1).

Two studies were recently published by the group of Frank Verreck evaluating innate and adaptive immunitiy induced following mucosal delivery of MTBVAC and BCG to the lungs in rhesus macaques [71,72]. Dijkman *et* al., [71] showed that pulmonary vaccination with MTBVAC in rhesus macaques resulted in more rapid induction of immune responses and broader antigenic specificity than BCG. Vierboom *et* al., [72] demonstrated that mucosal respiratory delivery of BCG or MTBVAC to rhesus macaques induced trained immunity, which was more efficient than standard intradermal vaccination, whereby MTBVAC and BCG were equailly effective. Moreover, mucosal vaccination enhanced innate cytokine production by blood- and bone marrow-derived monocytes associated with metabolic rewiring, typical of trained immunity. The findings by Vierboom *et al.*, support the innate immune stimulatory potential for MTBVAC and for live attenuated mycobacterial vaccines in general, with ability to enhance innate immune training via respiratory mucosal vaccine administration. Alternative delivery of new vaccines, like the aerosol route, with prior clinically demonstrated safety and immunogenicity, could be used for future eradication programmes.

Today MTBVAC remains the only live vaccine candidate in the clinical TB vaccine pipeline based on an attenuated *M. tuberculosis* strain. Considering the nature of MTBVAC, the vaccine conserves the entire T cell epitope repertoire described for MTBC pathogens including the major immunodominant antigens ESAT-6, CFP-10 and PPE68 which are absent in BCG due to the loss of RD1. Despite their small molecular weight, these three proteins are unusually immunogenic and contain 285 of the total 1603 epitopes described in *M. tuberculosis*, recognized by human MHC haplotypes [35]. In line with this observation, our preclinical studies published by Aguilo *et al.* [24] have demonstrated that immunity against ESAT-6 and CFP-10 conferred after MTBVAC vaccination remarkably correlates with improved vaccine efficacy relative to BCG. What is more, MTBVAC-conferred ESAT-6/CFP-10 reactogenicity important for improved protection in mice was shown to depend on host genetic background, demonstrating the exclusive capacity of the H-2 k (MHC II) haplotype of the C3H mouse strain, but not of the two commonly used mouse strains BALB/c and BL6/C57, to recognize the ESAT-6 and CFP-10-derived peptides. Our studies high-

Table 1
Non-specific effects of BCG and MTBVAC.

BCG	MTBVAC
First-line therapy for non–muscle-invasive bladder cancer. [69]	Therapeutic efficacy in preclinical model of bladder cancer. [65]
Trained immunity in human cells. [70]	Trained immunity in human cells (epigenetic and metabolic reprogramming of the cells from the innate immune system). [67]
Heterologous protection against lethal *Candida albicans* infection in mice. [70]	Heterologous protection against a lethal challenge with *Streptococcus pneumoniae* in an experimental murine model of pneumonia. [67]
Therapeutic efficacy against established asthma. [66]	Therapeutic efficacy against established asthma. [66]
Enhancing humoral and cellular immunity of DTaP vaccine (diphtheria, tetanus, and acellular pertussis) in mice. Ten-fold lower incidence of pertussis in countries using BCG and DTaP than countries using only DTaP. [68]	Enhancing humoral and cellular immunity of DTaP vaccine (diphtheria, tetanus, and acellular pertussis) in mice. [68]

light the importance of host genetics in vaccine induced protection. In humans, studies show that about 60–80% of individuals are able to respond to these two antigens [73,74], making us hypothesize that vaccine-induced reactogenicity to ESAT-6 and CFP-10 might confer improved protection in these individuals (as compared to BCG), what would have an evident impact on TB transmission. Conducting clinical trials in diverse geographical regions to account for differences in host genetic background would provide valuable insights for this hypothesis. In support of this hypothesis are prospective cohort studies of persons exposed to individuals with active TB indicating that latently infected people (reactive to ESAT-6 and CFP-10 stimulation) are more protected against re-infection than non-infected ones. Apart from host-genetic background, presence of environmental mycobacteria has also been shown to affect BCG-vaccine efficacy due to potential masking and blocking events [75]. This is especially important for new TB vaccines targeting age groups other than newborns. Considerations for conducting clinical trials in representative geographical latitudes, such as tropical regions where higher prevalence of environmental mycobacteria is observed versus those further away from the equator, where environmental mycobacteria infections are lower [75], should form part of a successful clinical development strategy of new TB vaccines.

More recently, MTBVAC vaccine efficacy was reapreaised in the context of the evolutionary genomics of *M. tuberculosis*, where MTBVAC variants constructed in clinical isolates belonging to lineages 2 (which includes Beijing strains) and 3 (L2 and L3) together with the original MTBVAC vaccine, derivative of L4, were compared against BCG in mouse models [76]. Of note L2, L3 and L4 are considered the three most geographically distributed lineages of the MTBC responsible for a large proportion of the TB burden worldwide today, as such the study served to see whether vaccine efficacy could be lineage specific. Safety studies in immunocompromised mice showed that MTBVAC-L2 was less attenuated than BCG Pasteur, whereas the original MTBVAC was found even more attenuated than BCG, and MTBVAC-L3 showed an intermediate phenotype. Efficacy studies in immunocompetent mice against L2, L3 and L4 aerosol challenge showed similar or superior protection as compared to BCG Pasteur for all three MTBVAC vaccines [76]. All these studies indicate that MTBVAC could protect clinically against the three modern TB lineages (L2, L3 and L4) that cause more than 95% of tuberculosis cases in humans and support the planned Phase 3 efficacy trials in agreement with regulatory requirements.

10. The need for new more reliable TB diagnostic tests

A question concerning conduct of community-wide Phase 3 efficacy trials with new TB vaccines is the availability of an adequate TB-diagnostic test which does not interfere with vaccine-induced immune responses. The Phase 1b trial of MTBVAC noted a dose-related QuantiFERON-TB-Gold (QFT) test conversion, however by day 360, a dose-related QFT reversion had occurred ranging from 100% in the lowest dose group to 43% in the highest dose group [61]. Importantly, QuantiFERON TB and ELISPOT-based (T. SPOT-TB) interferon gamma release assays (IGRAs) are the two commercially available test frequently employed by PIs in clinical trials for detecting *M. tberculosis* infection. However, both tests are based on detecting response to ESAT-6 and CFP-10 epitopes, two of the most immunodominant *M. tuberculosis* antigens, which are still present in MTBVAC and have been correlated with vaccine improved efficacy [24]. The lack of diversity in new TB-diagnostics that could aid in the efficacy evaluation of new TB vaccines is of great vaccine development and epidemiological importance. The evidence of immunogenicity of MTBVAC supports its progression into larger safety and efficacy trials, but also confounds interpretation of the current tests for *M. tuberculosis* infection, highlighting the need for stringent endpoint definition [61].

MTBVAC vaccine development efforts are focused on the discovery of a more effective TB vaccine that could help reverse TB incidence and mortality rates, and on the development of a vaccine-specific test for differentiation of MTBVAC and *M. tuberculosis* infection. As mentioned earlier, in order to have an effective vaccine, we need to understand immunity needed for TB protection, and in order to understand protective immunity (and have correlates of TB protection) we need an effective TB vaccine [15]. As in the case of COVID-19, the lack of a reliable diagnostic test did not hinder the development of different effective vaccination strategies that could save millions of lives. Once such effective vaccines are made available for mass vaccination campaigns, it would be easier to identify correlates of protection that could aid in the development of reliable diagnostic tests able to differentiate protection from infection and disease.

11. Final remarks

BCG has been in use for one hundred years now and has served to provide continous new insights into vaccination against TB [77], positioning it as true guide in the design and development of hundreds of new TB vaccine candidates in the last two and a half decades. MTBVAC is one of these candidates in the global TB vaccine pipeline. Based on a live-attenuated *M. tuberculosis* clinical isolate, MTBVAC's 25 years of vaccine discovery, construction and characterization have strictly abided by Pasteur principles, similar to BCG. Using BCG as an established referencegold standard for safety and protective efficacy, MTBVAC, which contains the whole antigen repertoire of *M. tuberculosis*, has demonstrated superior protective efficacy in mice, guinea pigs and non-human primates and similar safety as compared to BCG. Today MTBVAC is the first and only candidate of its nature in clinical development ready to move into Phase 3 efficacy trials in newborns in TB-endemic countries. Beyond conferring protection against TB, BCG is recognized for its ability to induce non-specific immunity to other heterologous diseases and all-cause mortality in the first months of life. Today, the WHO recognizes the importance that any new TB vaccine designed for administration at birth, should show similar non-specific benefits as BCG vía mechanisms of trainned immunity and/or cross-reactivity of adaptive immunine responses to other pathogens. Following the footsteps of BCG's application in different therapeutic settings, key recent studies provide strong support for MTBVAC's ability of inducing trainned immunity and confering non-specific heterologous protection similar to BCG. Research on alternative delivery routes of MTBVAC, like a clinically feasible aerosol route, could facilitate vaccine administration for long-term TB eradication programmes in post-licensure studies of new TB vaccines.

Declaration of Competing Interest

C.M., D.M., N.A. and J. G. are co-inventors on a patent on Tuberculosis Vaccine held by the University of Zaragoza and Biofabri. Biofabri is industrial partner of University of Zaragoza and the exclusive licensee and industrial and clinical developer of MTBVAC. The authors declare that they have no other known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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(at present RTC-2017-6379-1). European MTBVAC vaccine project is linked to the TB vaccine R&D program of the TuBerculosis Vaccine Initiative (TBVI)-governed TBVAC2020 Consortium, supported by the European Commission under the Horizon 2020 programme, grant agreement no. 643381. Phase 1b/2a in BCG-vaccinated adolescents and adults with and without prior *M. tuberculosis* infection is conducted in collaboration with IAVI and financed by funds from the INH and CDRMP of the United States government. The currently ongoing Phase 2 dose-defining clinical trial in newborns counts with funding from The European & Developing Countries Clinical Trials Partnership EDCTP2 "RIA-2016-V-1637" MTBVACN2. EDCTP has granted funding for the planned MTBVAC Phase 3 efficacy trial (now expected to commence in 2022) through "RIA2019S-2652" MTBVACN3 Efficacy trial: "MTBVAC in Newborns: Randomised, Double-blind Controlled Phase 3 Trial to evaluate the Efficacy, Safety and Immunogenicity of MTBVAC administered in healthy HIV unexposed uninfected and HIV exposed uninfected newborns in Tuberculosis-Endemic Regions of Sub-Saharan Africa."

Data Sharing Statement

The results referred to in the present review are based on published works that are publically available and traceable on PubMed database.

References

[1] WHO. World Health Organization Global Tuberculosis Report 2020. Geneva: World Health Organization; 2020. Licence: CC BY-NC-SA 3.0 IGO.2020.
[2] https://www.who.int/teams/health-product-and-policy-standards/standards-and-specifications/vaccines-quality/bcg. BCG Vaccine.
[3] WHO. World Health Organization model list of essential medicines: 21st list 2019. Geneva: World Health Organization. hdl:10665/325771. . 2019.
[4] WHO. BCG vaccines: WHO position paper. Weekly Epidemiological Record. 8 (93): 73–96. 23 February 2018 Geneva, Switzerland2018.
[5] Colditz GA, Berkey CS, Mosteller F, Brewer TF, Wilson ME, Burdick E, et al. The efficacy of bacillus Calmette-Guerin vaccination of newborns and infants in the prevention of tuberculosis: meta-analyses of the published literature. Pediatrics 1995;96:29–35.
[6] Aronson NE, Santosham M, Comstock GW, Howard RS, Moulton LH, Rhoades ER, et al. Long-term efficacy of BCG vaccine in American Indians and Alaska Natives: A 60-year follow-up study. JAMA 2004;291:2086–91.
[7] Abubakar I, Pimpin L, Ariti C, Beynon R, Mangtani P, Sterne JA, et al. Systematic review and meta-analysis of the current evidence on the duration of protection by bacillus Calmette-Guerin vaccination against tuberculosis. Health Technology Assessment 2013;17(1–372):v–vi.
[8] Nguipdop-Djomo P, Heldal E, Rodrigues LC, Abubakar I, Mangtani P. Duration of BCG protection against tuberculosis and change in effectiveness with time since vaccination in Norway: a retrospective population-based cohort study. Lancet Infectious Diseases 2016;16:219–26.
[9] WHO. WHO Coronavirus Disease (COVID-19) Dashboard. https://covid19.who.int/.
[10] Cilloni L, Fu H, Vesga JF, Dowdy D, Pretorius C, Ahmedov S, et al. The potential impact of the COVID-19 pandemic on the tuberculosis epidemic a modelling analysis. EClinicalMedicine. 2020;28:100603.
[11] WHO. WHO End TB Strategy. WHO/CDS/TB/2018.29 – © World Health Organization 2018. , https://www.who.int/tb/End_TB_brochure.pdf?ua=1.
[12] WHO. WHO Preferred Product Characteristics for New Tuberculosis Vaccines. Ordering code: WHO/IVB/18.06 2018.
[13] www.tbvi.eu. TuBerculosis Vaccine Initiative (TBVI).
[14] Martin C, Aguilo, N., Marinova, D., & Gonzalo-Asensio, J. . Update on TB vaccine pipeline. . Applied Sciences (Switzerland), 10(7) http://doiorg/103390/app10072632. 2020.
[15] Douglas Young P. Research and develoment of new TB vaccines. International TB Vaccine Symposium Paraninfo Building, University of Zaragoza, Zaragoza, Spain. Sponsors/Organizers: Fundación Ramón Areces, TBVI, University of Zaragoza3-4 June 2010.
[16] Pelicic V, Jackson M, Reyrat JM, Jacobs Jr WR, Gicquel B, Guilhot C. Efficient allelic exchange and transposon mutagenesis in Mycobacterium tuberculosis. Proc Natl Acad Sci U S A. 1997;94:10955–60.
[17] Guilhot C, Otal I, Van Rompaey I, Martin C, Gicquel B. Efficient transposition in mycobacteria: construction of Mycobacterium smegmatis insertional mutant libraries. Journal of Bacteriology 1994;176:535–9.
[18] Gicquel B. Towards new mycobacterial vaccines. Developments in Biological Standardization 1994;82:171–8.
[19] Guilhot C, Gicquel B, Davies J, Martin C. Isolation and analysis of IS6120, a new insertion sequence from Mycobacterium smegmatis. Molecular Microbiology 1992;6:107–13.
[20] Martin C, Timm J, Rauzier J, Gomez-Lus R, Davies J, Gicquel B. Transposition of an antibiotic resistance element in mycobacteria. Nature 1990;345:739–43.
[21] Ranes MG, Rauzier J, Lagranderie M, Gheorghiu M, Gicquel B. Functional analysis of pAL5000, a plasmid from Mycobacterium fortuitum: construction of a "mini" mycobacterium-Escherichia coli shuttle vector. Journal of Bacteriology 1990;172:2793–7.
[22] Kamath AT, Fruth U, Brennan MJ, Dobbelaer R, Hubrechts P, Ho MM, et al. New live mycobacterial vaccines: the Geneva consensus on essential steps towards clinical development. Vaccine. 2005;23:3753–61.
[23] Arbues A, Aguilo JI, Gonzalo-Asensio J, Marinova D, Uranga S, Puentes E, et al. Construction, characterization and preclinical evaluation of MTBVAC, the first live-attenuated M. tuberculosis-based vaccine to enter clinical trials. Vaccine. 2013;31:4867–73.
[24] Aguilo N, Gonzalo-Asensio J, Alvarez-Arguedas S, Marinova D, Gomez AB, Uranga S, et al. Reactogenicity to major tuberculosis antigens absent in BCG is linked to improved protection against Mycobacterium tuberculosis. Nature Communications 2017;8:16085.
[25] Clark S, Lanni F, Marinova D, Rayner E, Martin C, Williams A. Revaccination of Guinea Pigs With the Live Attenuated Mycobacterium tuberculosis Vaccine MTBVAC Improves BCG's Protection Against Tuberculosis. Journal of Infectious Diseases 2017;216:525–33.
[26] White AD, Sibley L, Sarfas C, Morrison A, Gullick J, Clark S, et al. MTBVAC vaccination protects rhesus macaques against aerosol challenge with M. tuberculosis and induces immune signatures analogous to those observed in clinical studies. npj Vaccines 2021;6:4..
[27] Marinova D, Gonzalo-Asensio J, Aguilo N, Martin C. MTBVAC from discovery to clinical trials in tuberculosis-endemic countries. Expert Rev Vaccines. 2017;16:565–76.
[28] Brosch R, Gordon SV, Marmiesse M, Brodin P, Buchrieser C, Eiglmeier K, et al. A new evolutionary scenario for the Mycobacterium tuberculosis complex. Proc Natl Acad Sci U S A. 2002;99:3684–9.
[29] Boritsch EC. Brosch R. Evolution of Mycobacterium tuberculosis: New Insights into Pathogenicity and Drug Resistance. Microbiol Spectr; 2016. p. 4.
[30] Brosch R, Gordon SV, Garnier T, Eiglmeier K, Frigui W, Valenti P, et al. Genome plasticity of BCG and impact on vaccine efficacy. Proc Natl Acad Sci U S A. 2007;104:5596–601.
[31] Comas I, Chakravartti J, Small PM, Galagan J, Niemann S, Kremer K, et al. Human T cell epitopes of Mycobacterium tuberculosis are evolutionarily hyperconserved. Nature Genetics 2010;42:498–503.
[32] Yruela I, Contreras-Moreira B, Magalhaes C, Osorio NS, Gonzalo-Asensio J. Mycobacterium tuberculosis Complex Exhibits Lineage-Specific Variations Affecting Protein Ductility and Epitope Recognition. Genome Biol Evol. 2016;8:3751–64.
[33] Zhang W, Zhang Y, Zheng H, Pan Y, Liu H, Du P, et al. Genome sequencing and analysis of BCG vaccine strains. PLoS ONE 2013;8:e71243.
[34] Copin R, Coscolla M, Efstathiadis E, Gagneux S, Ernst JD. Impact of in vitro evolution on antigenic diversity of Mycobacterium bovis bacillus Calmette-Guerin (BCG). Vaccine. 2014;32:5998–6004.
[35] Gonzalo-Asensio J, Marinova D, Martin C, Aguilo N. MTBVAC: Attenuating the Human Pathogen of Tuberculosis (TB) Toward a Promising Vaccine against the TB Epidemic. Frontiers in Immunology 2017;8:1803.
[36] Copin R, Wang X, Louie E, Escuyer V, Coscolla M, Gagneux S, et al. Within Host Evolution Selects for a Dominant Genotype of Mycobacterium tuberculosis while T Cells Increase Pathogen Genetic Diversity. PLoS Pathogens 2016;12:e1006111.
[37] Zhang L, Ru HW, Chen FZ, Jin CY, Sun RF, Fan XY, et al. Variable Virulence and Efficacy of BCG Vaccine Strains in Mice and Correlation With Genome Polymorphisms. Molecular Therapy 2016;24:398–405.
[38] Gonzalo-Asensio J, Malaga W, Pawlik A, Astarie-Dequeker C, Passemar C, Moreau F, et al. Evolutionary history of tuberculosis shaped by conserved mutations in the PhoPR virulence regulator. Proc Natl Acad Sci U S A. 2014;111:11491–6.
[39] Iglesias MJ, Ibarz D, Cebollada A, Comin J, Jimenez MS, Vazquez MC, et al. The value of the continuous genotyping of multi-drug resistant tuberculosis over 20 years in Spain. Scientific Reports 2020;10:20433.
[40] Rivero A, Marquez M, Santos J, Pinedo A, Sanchez MA, Esteve A, et al. High rate of tuberculosis reinfection during a nosocomial outbreak of multidrug-resistant tuberculosis caused by Mycobacterium bovis strain B. Clinical Infectious Diseases 2001;32:159–61.
[41] Sagasti S, Millan-Lou MI, Soledad Jimenez M, Martin C, Samper S. In-depth analysis of the genome sequence of a clinical, extensively drug-resistant Mycobacterium bovis strain. Tuberculosis (Edinb). 2016;100:46–52.
[42] Gonzalo-Asensio J, Perez I, Aguilo N, Uranga S, Pico A, Lampreave C, et al. New insights into the transposition mechanisms of IS6110 and its dynamic distribution between Mycobacterium tuberculosis Complex lineages. PLoS Genetics 2018;14:e1007282.
[43] Perez E, Samper S, Bordas Y, Guilhot C, Gicquel B, Martin C. An essential role for phoP in Mycobacterium tuberculosis virulence. Molecular Microbiology 2001;41:179–87.
[44] Stucki D, Brites D, Jeljeli L, Coscolla M, Liu Q, Trauner A, et al. Mycobacterium tuberculosis lineage 4 comprises globally distributed and geographically restricted sublineages. Nature Genetics 2016;48:1535–43.

[45] Gonzalo-Asensio J, Mostowy S, Harders-Westerveen J, Huygen K, Hernandez-Pando R, Thole J, et al. PhoP: a missing piece in the intricate puzzle of Mycobacterium tuberculosis virulence. PLoS ONE 2008;3:e3496.
[46] Solans L, Aguilo N, Samper S, Pawlik A, Frigui W, Martin C, et al. A specific polymorphism in Mycobacterium tuberculosis H37Rv causes differential ESAT-6 expression and identifies WhiB6 as a novel ESX-1 component. Infection and Immunity 2014;82:3446–56.
[47] Solans L, Gonzalo-Asensio J, Sala C, Benjak A, Uplekar S, Rougemont J, et al. The PhoP-dependent ncRNA Mcr7 modulates the TAT secretion system in Mycobacterium tuberculosis. PLoS Pathogens 2014;10:e1004183.
[48] Chesne-Seck ML, Barilone N, Boudou F, Gonzalo Asensio J, Kolattukudy PE, Martin C, et al. A point mutation in the two-component regulator PhoP-PhoR accounts for the absence of polyketide-derived acyltrehaloses but not that of phthiocerol dimycocerosates in Mycobacterium tuberculosis H37Ra. Journal of Bacteriology 2008;190:1329–34.
[49] Gonzalo Asensio J, Maia C, Ferrer NL, Barilone N, Laval F, Soto CY, et al. The virulence-associated two-component PhoP-PhoR system controls the biosynthesis of polyketide-derived lipids in Mycobacterium tuberculosis. Journal of Biological Chemistry 2006;281:1313–6.
[50] Frigui W, Bottai D, Majlessi L, Monot M, Josselin E, Brodin P, et al. Control of M. tuberculosis ESAT-6 secretion and specific T cell recognition by PhoP. PLoS Pathogens 2008;4:e33..
[51] Walker KB, Brennan MJ, Ho MM, Eskola J, Thiry G, Sadoff J, et al. The second Geneva Consensus: Recommendations for novel live TB vaccines. Vaccine. 2010;28:2259–70.
[52] Cox JS, Chen B, McNeil M, Jacobs Jr WR. Complex lipid determines tissue-specific replication of Mycobacterium tuberculosis in mice. Nature 1999;402:79–83.
[53] Trivedi OA, Arora P, Vats A, Ansari MZ, Tickoo R, Sridharan V, et al. Dissecting the mechanism and assembly of a complex virulence mycobacterial lipid. Molecular Cell 2005;17:631–43.
[54] Kirksey MA, Tischler AD, Simeone R, Hisert KB, Uplekar S, Guilhot C, et al. Spontaneous phthiocerol dimycocerosate-deficient variants of Mycobacterium tuberculosis are susceptible to gamma interferon-mediated immunity. Infection and Immunity 2011;79:2829–38.
[55] Camacho LR, Constant P, Raynaud C, Laneelle MA, Triccas JA, Gicquel B, et al. Analysis of the phthiocerol dimycocerosate locus of Mycobacterium tuberculosis. Evidence that this lipid is involved in the cell wall permeability barrier. Journal of Biological Chemistry 2001;276:19845–54.
[56] Augenstreich J, Arbues A, Simeone R, Haanappel E, Wegener A, Sayes F, et al. ESX-1 and phthiocerol dimycocerosates of Mycobacterium tuberculosis act in concert to cause phagosomal rupture and host cell apoptosis. Cellular Microbiology 2017;19.
[57] Infante E, Aguilar LD, Gicquel B, Pando RH. Immunogenicity and protective efficacy of the Mycobacterium tuberculosis fadD26 mutant. Clinical and Experimental Immunology 2005;141:21–8.
[58] Aguilo N, Uranga S, Marinova D, Monzon M, Badiola J, Martin C. MTBVAC vaccine is safe, immunogenic and confers protective efficacy against Mycobacterium tuberculosis in newborn mice. Tuberculosis (Edinb). 2016;96:71–4.
[59] Martin C, Williams A, Hernandez-Pando R, Cardona PJ, Gormley E, Bordat Y, et al. The live Mycobacterium tuberculosis phoP mutant strain is more attenuated than BCG and confers protective immunity against tuberculosis in mice and guinea pigs. Vaccine. 2006;24:3408–19.
[60] Spertini F, Audran R, Chakour R, Karoui O, Steiner-Monard V, Thierry AC, et al. Safety of human immunisation with a live-attenuated Mycobacterium tuberculosis vaccine: a randomised, double-blind, controlled phase I trial. Lancet Respir Med. 2015;3:953–62.
[61] Tameris M, Mearns H, Penn-Nicholson A, Gregg Y, Bilek N, Mabwe S, et al. Live-attenuated Mycobacterium tuberculosis vaccine MTBVAC versus BCG in adults and neonates: a randomised controlled, double-blind dose-escalation trial. Lancet Respir Med. 2019;7:757–70.
[62] Prentice S, Nassanga B, Webb EL, Akello F, Kiwudhu F, Akurut H, et al. BCG-induced non-specific effects on heterologous infectious disease in Ugandan neonates: an investigator-blind randomised controlled trial. Lancet Infectious Diseases 2021.
[63] Benn CS, Netea MG, Selin LK, Aaby P. A small jab - a big effect: nonspecific immunomodulation by vaccines. Trends in Immunology 2013;34:431–9.
[64] Arts RJ, Carvalho A, La Rocca C, Palma C, Rodrigues F, Silvestre R, et al. Immunometabolic Pathways in BCG-Induced Trained Immunity. Cell Rep. 2016;17:2562–71.
[65] Alvarez-Arguedas S, Uranga S, Martin M, Elizalde J, Gomez AB, Julian E, et al. Therapeutic efficacy of the live-attenuated Mycobacterium tuberculosis vaccine, MTBVAC, in a preclinical model of bladder cancer. Transl Res. 2018;197:32–42.
[66] Tarancon R, Mata E, Uranga S, Gomez AB, Marinova D, Otal I, et al. Therapeutic efficacy of pulmonary live tuberculosis vaccines against established asthma by subverting local immune environment. EBioMedicine. 2021;64:103186.
[67] Tarancon R, Dominguez-Andres J, Uranga S, Ferreira AV, Groh LA, Domenech M, et al. New live attenuated tuberculosis vaccine MTBVAC induces trained immunity and confers protection against experimental lethal pneumonia. PLoS Pathogens 2020;16:e1008404.
[68] Esther Broset JP-S, Alex I. Kanno, Nacho Aguilo, Ana Isabel Dacosta, Irene Rivero-Calle, Jesus Gonzalo-Asensio, Camille Locht, Luciana C.C. Leite, Carlos Martin, Federico Martinón-Torres. BCG vaccination improves DTaP immune responses in mice and is associated with lower pertussis incidence in ecological epidemiological studies. EBioMedicine 65 (2021) 103254 https://doiorg/101016/jebiom2021103254. 2021.
[69] Morales A, Eidinger D, Bruce AW. Intracavitary Bacillus Calmette-Guerin in the treatment of superficial bladder tumors. Journal of Urology 1976;116:180–3.
[70] Kleinnijenhuis J, Quintin J, Preijers F, Joosten LA, Ifrim DC, Saeed S, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A. 2012;109:17537–42.
[71] Dijkman K, Aguilo N, Boot C, Hofman SO, Sombroek CC, Vervenne RAW, et al. Pulmonary MTBVAC vaccination induces immune signatures previously correlated with prevention of tuberculosis infection. Cell Rep Med. 2021;2:100187.
[72] Vierboom MPM, Dijkman K, Sombroek CC, Hofman SO, Boot C, Vervenne RAW, et al. Stronger induction of trained immunity by mucosal BCG or MTBVAC vaccination compared to standard intradermal vaccination. Cell Rep Med. 2021;2:100185.
[73] Lindestam Arlehamn CS, McKinney DM, Carpenter C, Paul S, Rozot V, Makgotlho E, et al. A Quantitative Analysis of Complexity of Human Pathogen-Specific CD4 T Cell Responses in Healthy M. tuberculosis Infected South Africans. PLoS Pathog. 2016;12:e1005760.
[74] Millington KA, Fortune SM, Low J, Garces A, Hingley-Wilson SM, Wickremasinghe M, et al. Rv3615c is a highly immunodominant RD1 (Region of Difference 1)-dependent secreted antigen specific for Mycobacterium tuberculosis infection. Proc Natl Acad Sci U S A. 2011;108:5730–5.
[75] Barreto ML, Pilger D, Pereira SM, Genser B, Cruz AA, Cunha SS, et al. Causes of variation in BCG vaccine efficacy: examining evidence from the BCG REVAC cluster randomized trial to explore the masking and the blocking hypotheses. Vaccine. 2014;32:3759–64.
[76] Perez I, Uranga S, Sayes F, Frigui W, Samper S, Arbues A, et al. Live attenuated TB vaccines representing the three modern Mycobacterium tuberculosis lineages reveal that the Euro-American genetic background confers optimal vaccine potential. EBioMedicine. 2020;55:102761.
[77] Scriba TJ, Mizrahi V. Renewing the Fight Against TB with an Old Vaccine. Cell 2020;180:829–31.
[78] Sakula A. BCG: who were Calmette and Guerin?. Thorax 1983;38:806–12.