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Preface

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Title

Neonatal vaccination with Bacille Calmette-Guérin strains: Effects in infancy

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Cover page illustration Mike Berendsen. **Print** Grafisk Center, SDU.

Contents

Preface

The work presented in this Ph.D. thesis was conducted between January 2015 and April 2020 and involved two years and ten months of fieldwork in Guinea-Bissau, on top of a research year I had in 2012. Moving to Guinea-Bissau changes you. It is as if those that went down that road can be recognized from far away by a distinct charm and charisma, at least that is the sensation I get. Will I develop such a glow, too? My time in Guinea-Bissau certainly has changed my life, and I am forever grateful and indebted for the opportunities and trust I have been offered. Aside from the chance to contribute to highly relevant research, I struck gold in Bissau and married the girl next door, who has engifted me not only with her company but also with our beautiful, intelligent, and curly-haired daughter. I arrived as a just-graduated doctor on a motorcycle and now hand in this thesis as a married father. But my time in Bissau has undoubtedly also been a rollercoaster ride with incredible highs and lows, and I had myself to blame when things did not go my way.

In a setting with limited access to the most essential elements of health care, one might speculate whether the relevance to clinical practice in Denmark is negligible and that one would become a better doctor by spending time in a Danish emergency room. While I acknowledge that there are skills I need to catch up on, I am confident that working in Bissau has made me a *better* doctor, which I can self-confirm from my biased admiration of the cohort of Danish doctors that "repatriated" from Bissau. They never lost neither the Bissau glow nor their passion for improving health care standards for all and reduce inequality in health.

I am grateful to many unique individuals that I have met over the years, among others:

Vu for your friendship and good advice when I first arrived in Bissau, including advising me to find a small motorcycle to alleviate transport necessities in Bissau. Gabriel for teaching me how to speak Creole, ride a bike, improving my understanding of the Guinean culture and for being a magnificent study supervisor at the Maternity Ward.

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Ádám, the Hungarian farmer who participated in the Budapest-Bamako caravan in an old Mercedes 190D and went to fetch gasoline for me in the dark outskirts of Tangiers when I ran it dry and still did not know how to turn the fuel tap on for the reserve tank. My fellow Africa Twin rider Jason Andean for providing four pages of instructions on preparing the XRV750 for crossing the Sahara, and for your unique friendship, inspirational and a kind yet robust approach on how to safely complete the trip, along with fellow riders Mark Jeunnette and David Armstrong a.k.a. Special Needs. I am forever grateful for riding with you. The two mechanical engineers from MIT deserves mentioning too, for first insisting on refueling and going to the beach rather than heading straight to Nouakchott, then mistaking *essence* for *gazole* while the doctor in the pack (me) was suggesting that the gasoline looked strange. Since then, I have been able to handle any crisis with stoic calmness, because little can be worse than standing in the middle of the desert in Northern Mauritania with 4 stalled 220-kilo bikes due to diesel-congested carburetors.

This thesis would never have had the quality it deserves had it not been for Ivan, who served as the study supervisor and main data entry clerk for all five RCTs that contributed data. A unique and orderly attention to details, effectiveness, due diligence, hard work, and at the same time, putting forward a friendly, relaxed mindset has made it the greatest pleasure to conduct projects and share office space with you. No gift can express my gratitude to you.

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The capacity to successfully conduct large-scale RCTs in Bissau with adequate follow-up was built and maintained over many years with the contribution of many researchers. The well-conducted RCTs that form the basis of this thesis were thanks to substantial contributions and capacity-building in Bissau done by Sofie, Kristoffer, Helle, Najaaraq, Adam and Amabélia with contributions also from many research year students. Thanks to the guards at the Institute of Public Health (Claus, Bendt, and Martin) for always bringing a cheerful smile and keeping my spirits up when I was once again working at very odd hours. Ziggi and Carsten for good vibes around the institute and fun table tennis matches. Susanne & Tina for taking care of the practicalities so that the researchers can do research. And thanks to all the scientists that I have co-authored papers with.

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List of thesis papers

I. F Schaltz-Buchholzer, HN Frankel, CS Benn: *The real-life number of neonatal doses of Bacille Calmette-Guérin vaccine in a 20-dose vial.* Glob Health Action. 2017;10(1):1-4. doi: 10.1080/16549716.2017.1267964.

II. F Schaltz-Buchholzer, S Biering-Sørensen, N Lund, I Monteiro, P Umbasse, AB Fisker, A Andersen, A Rodrigues, P Aaby, CS Benn: *Early BCG Vaccination, Hospitalizations, and Hospital Deaths: Analysis of a Secondary Outcome in 3 Randomized Trials from Guinea-Bissau.* J Infect Dis. 2019 Jan 29;219(4):624-632. doi: 10.1093/infdis/jiy544.

III. F Schaltz-Buchholzer, M Bjerregaard-Andersen, CB Øland, C Golding, EB Stjernholm, I Monteiro, P Aaby, CS Benn: *Early vaccination with BCG-Denmark or BCG-Japan versus BCG-Russia to healthy newborns in Guinea-Bissau: A randomized controlled trial.* Clin Infect Dis. 2019 Nov 3. pii: ciz1080. doi: 10.1093/cid/ciz1080.

IV. F Schaltz-Buchholzer, M Berendsen, A Roth, KJ Jensen, M Bjerregaard-Andersen, MJ Sørensen, I Monteiro, P Aaby, CS Benn: *BCG skin reactions by 2 months of age are associated with better survival in infancy: A prospective observational study* (Submitted, BMJ Global Health)

V. F Schaltz-Buchholzer, S Biering-Sørensen, LCJ de Bree, A Roth, CAG Timmermann, I Monteiro, P Aaby, CS Benn: *Neonatal Bacille Calmette-Guérin vaccination and tuberculin skin test reactions at 2- and 6-months: effects on mortality up to 1 year of age.* (Manuscript)

Additional scientific contributions not included in the thesis

CL Martins, CS Benn, A Andersen, C Balé, **F Schaltz-Buchholzer**, VA Do, A Rodrigues, P Aaby, H Ravn, H Whittle, ML Garly: *A randomized trial of a standard dose of Edmonston-Zagreb measles vaccine given at 4.5 months of age: effect on total hospital admissions.* J Infect Dis. 2014 Jun 1;209(11):1731-8.

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CS Benn, A Roth, ML Garly, AB Fisker, **F Schaltz-Buchholzer**, CAG Timmermann, M Berendsen, Peter Aaby: *BCG-scarring and improved child survival: A combined analysis of studies of BCG-scarring.* In press, Journal of Internal Medicine.

CN Golding, **F Schaltz-Buchholzer**, L Sanca, C Clipet-Jensen, C Stabell Benn, N Au, K Chipperfield, TR Kollmann, NA Amenyogbe: *Feasibility of manual white blood cell counts as predictor of neonatal sepsis in a low-resource setting.* In press, TRSTMH.

B Brook, DJ Harbeson, CP Shannon, R Ben-Othman, B Ca, D He, F Francis, J Huang, N Varankovich, A Liu, W Bao, M Bjerregaard-Andersen, **F Schaltz-Buchholzer**, L Sanca, CN Golding, KL Larsen, O Levy, B Kampmann, The EPIC Consortium, JL W, F Shann, P Aaby, CS Benn, S Tebbutt, N Amenyogbe, TR Kollmann. *BCG-vaccination induced emergency granulopoiesis provides rapid protection from neonatal sepsis*. In press, Sci Transl Med.

List of abbreviations

aMRR, adjusted Mortality Rate Ratio

BCG, Bacille Calmette-Guérin vaccine

BHP, Bandim Health Project

CFR, Case-Fatality Rate

CFU, Colony-Forming Unit

CNS, Central Nervous System

DSMB, Data and Safety Monitoring Board

DTP, Diphtheria-Tetanus-Pertussis vaccine

EG, Emergency Granulopoiesis

EONS, Early-Onset Neonatal Sepsis

G-CSF, Granulocyte Colony Stimulating Factor

HDSS, Health Demographic Surveillance System

HNSM, Hospital National Simão Mendes

HR, Hazard Ratio

LBW, Low Birth Weight

LMIC, Low and Middle-Income Countries

MD, Mean Difference

MRC, Medical Research Council

MUAC, Mid-Upper Arm Circumference

MV, Measles Vaccine

NICU, Neonatal Intensive Care Unit

NNV, Number Needed to Vaccinate

NSEs, Non-Specific Effects

OPV, Oral Polio Vaccine

OR, Odds Ratio

PPD, Purified Protein Derivative (synonym: TST)

PR, Prevalence Ratio

PYRS, Person-Years

RCT, Randomized Controlled Trial

RR, Risk Ratio

SD, Standard Deviation

SSI, Statens Serum Institut

TB, Tuberculosis

TST, Tuberculin Skin Test (synonym: PPD)

TU, Tuberculin Units

VE, Vaccine Efficacy

VLBW, Very Low Birth Weight

WHO, World Health Organization

Explanatory terms

Neonatal period: First 28 days of life.

Infant period: First year of life.

RCT I: Small RCT of early BCG versus delayed BCG provided to neonates with low weight, conducted from 2002-04 at three health centers in the BHP HDSS.

RCT II: RCT of early BCG versus delayed BCG provided to neonates with low weight conducted at three health centers in the BHP HDSS and at the HNSM Maternity Ward between 2004-2008.

RCT III: RCT of early BCG versus delayed BCG provided to neonates with low weight conducted at three health centers in the BHP HDSS and the HNSM Maternity Ward between 2008-2013.

RCT IV: RCT of immediate BCG versus BCG-at-discharge among infants admitted to the nursery at the HNSM Maternity Ward, conducted between 2013-2017.

RCT V: RCT of BCG-Denmark versus BCG-Russia and BCG-Japan versus BCG-Russia provided at discharge from the Maternity Ward of HNSM between 2014-2017.

Summary

This Ph.D. thesis investigated the importance of BCG vaccine strains for overall morbidity and mortality.

Vaccination with Bacille Calmette-Guérin at birth is recommended in countries where TB and/or leprosy are endemic, and >120 million infants are vaccinated per year. While BCG provides good protection against the most severe forms of TB, the protective efficacy against pulmonary TB ranges between 0-80%. There is accumulating evidence from observational studies and RCTs indicating that the Danish strain of BCG has beneficial NSEs, reducing all-cause mortality by 30-50%. The effect on the risk of hospitalizations had not been assessed prior to this thesis. Among BCG-vaccinated infants, those that develop a BCG skin reaction and/or a tuberculin response have a 30-50% lower mortality than non-reactors. The characteristics of the BCG skin reaction response types had not been investigated prior to this thesis. BCG is a live-attenuated vaccine produced at a host of different laboratories around the world. The vaccine is not a standardized and pharmacologically well-defined product and it has been suggested that different BCG strains should be considered as different vaccines. Different BCG formulations are genetically divergent due to the accumulation of mutations and differences in laboratory production techniques affecting the absolute number of bacteria and the ratio of live-to-dead bacteria. It is unknown whether BCG strains are bioequivalent. In 2018, 184.5 million doses of the WHO-prequalified BCG strains were distributed. The global market share in 2018 for the genetically related BCG-Denmark and BCG-Green Signal was 24%, BCG-Bulgaria 20%, BCG-Japan 9% and BCG-Russia 41%. None of these major BCG strains have been evaluated against each other in a randomized trial. Given the widespread use of BCG and its substantial NSEs, we investigated the effects on overall mortality and morbidity and on markers of protection against TB and a well-functioning immune system of neonatal vaccination with different BCG strains in Guinea-Bissau.

We had the following hypotheses:

Hypothesis A. *BCG vaccines possess beneficial, non-specific effects related to mortality and morbidity. Since BCG strains are phenotypically distinct, the size of these effects might be different. Compared with BCG-Russia, vaccination with BCG-Denmark and BCG-Japan is associated with fewer admissions, lower in-hospital case-fatality and fewer deaths.*

Hypothesis B. *The proportion of children having a BCG scar and a positive PPD response is 30% lower among children vaccinated with BCG-Russia compared with BCG-Denmark and BCG-Japan.*

Hypothesis C. *BCG vaccines are renowned for their safety. Since BCG-Denmark and BCG-Japan are probably more potent vaccines, they might be associated with a higher rate of adverse events when compared to BCG-Russia.*

During the data collection for this Ph.D., some new ideas evolved. From observations and personal experience of diverse BCG reactions, the Ph.D. student decided to study the importance of the BCG reaction type and size of early BCG skin reactions and the effects of

these reaction characteristics on subsequent all-cause mortality. And due to input from our vaccinators, we compared two BCG strains with regard to the content of neonatal BCG doses in a vaccine vial.

BCG is shipped as a vial of freeze-dried bacteria to be reconstituted with a vial containing 1 ml diluent before use. According to manufacturers, these vials contain 20 BCG doses of 0.05 ml for infants or ten doses of 0.1 ml each for children > one year of age and adults. It would, however, only be possible to withdraw and administer these doses if there was zero wastage of liquid in the syringes used for reconstitution and vaccination, as well as in the diluent and vaccine bottles.

In Paper I, we tested whether it is possible to withdraw 20 neonatal doses from vials of BCG-Russia and BCG-Denmark. Our experienced vaccinators could only withdraw 13 to 15 doses from both types of vials. This has important implications as vaccine wastage is calculated based on the assumption that the content is 20 doses; hence, wastage of BCG is overestimated, and this leads to unnecessary delays in BCG vaccination due to restrictive vial policies imposed to reduce vaccine wastage.

In Paper II, we supplemented the data on the effect of BCG-Denmark on mortality with data on its effect on hospital admission incidence and in-hospital mortality across three RCTs (6,583 infants). BCG-Denmark did not affect the risk of hospitalization but reduced the severity of disease, the neonatal BCG/no BCG CFR RR being 0.58 (0.35-0.94). Early-BCG especially reduced the risk of fatal neonatal sepsis, the CFR RR being 0.46 (0.22-0.98).

In Paper III, we investigated the effects of providing different BCG strains on morbidity, mortality, BCG skin reactions, tuberculin reactions, and adverse events (ipsilateral lymphadenitis). We conducted a large-scale RCT evaluating BCG-Denmark versus BCG-Russia which was scheduled to enroll 12,000 neonates at the HNSM Maternity Ward. Due to a production halt occurring in 2015 at the BCG manufacturing unit of Statens Serum Institut in Copenhagen, our last stock of BCG-Denmark was depleted by July 2016, almost midway in the trial. We had sought the necessary permits to exchange BCG-Denmark with BCG-Japan for the remainder of the trial and reached 12,000 inclusions in October 2017. Our data indicated no significant differences in morbidity nor mortality by six weeks of age associated with different strains of BCG, but the trial had limited power due to the change in intervention, especially for the mortality comparison. There was, however, somewhat fewer deaths associated with BCG-Japan, the Japan/Russia MRR being 0.71 (0.43-1.19). BCG-Denmark and BCG-Japan are likely more immunogenic than BCG-Russia since they were associated with more and larger early BCG skin reactions, tuberculin reactions and adverse events. These strain differences might have important implications both for the protection conferred against TB (specific effect) and for overall health (non-specific effects).

The indications of differences are currently being evaluated in a follow-up trial. We found a significant difference in the proportion of infants with a BCG scar, but much less than the hypothesized 30% difference. BCG-Denmark and BCG-Japan tended to be associated with more adverse events compared to BCG-Russia, but we lacked power to provide definitive conclusions.

There was a high BCG scar prevalence for all BCG strains. The proportion with a positive TST response to 2 TU stimulation was 65% lower for BCG-Russia compared to BCG-Denmark and 46% lower for BCG-Russia compared to BCG-Japan. The incidence of adverse events (lymphadenitis) among infants with home follow-up was 1.0% for BCG-Denmark versus 0.2% for BCG-Russia (p=0.12) and 1.2% for BCG-Japan versus 0.0% for BCG-Russia (p=0.03).

In Paper IV, we studied the idea that BCG reaction characteristics affect all-cause mortality and that the BCG strain administered is one of the main determinants of these characteristics. In a large cohort of 6,012 infants that had received BCG within one week of birth, 97% (5,804/6,012) had a BCG skin reaction by two months and the 2-12-month mortality risk among reactors was 1.9% versus 4.8% for non-reactors. Reacting to BCG versus not reacting was associated with an aMRR of 0.50 (0.26-0.96). Interestingly, the mortality risk was inversely correlated with the size of the BCG skin reaction, the risk being 2.9% for infants with small reactions, 1.8% for medium reactions, and 0.8% for large reactions. The corresponding large/small reaction aMRR was 0.35 (0.20-0.63), and there was a linear trend of decreasing mortality with increasing reaction size (p for trend<0.001). We concluded that the main determinant for both developing a BCG skin reaction and the size of the reaction is the strain of BCG utilized; BCG-Denmark and BCG-Japan are associated with more and larger reactions than BCG-Russia.

In Paper V, we evaluated the effect of having a TST reaction (defined as >1mm) at 2- and 6-months of age versus not having a reaction on all-cause mortality up to 12 months of age. The cohort of infants had received BCG during the neonatal period. Having a TST reaction at two months versus no reaction was associated with a 2-12-month aMRR of 0.34 (0.12-0.94), while at six months, the aMRR was 0.66 (0.15-2.92). We conducted a meta-analysis of the available studies and across four cohorts from Guinea-Bissau, having a 2-month tuberculin reaction versus no reaction was associated with an aMRR of 0.56 (0.38-0.83). Similarly, the effect on mortality of having a 6-month tuberculin reaction versus no reaction was an aMRR of 0.63 (0.39-1.00) across four cohorts. The main determinants for having a tuberculin reaction by six months were large post-vaccination wheals (receiving a large dose of BCG) and being vaccinated with BCG-Denmark or BCG-Japan rather than BCG-Russia.

In conclusion, our findings support that BCG has marked beneficial NSEs that enhance survival substantially. These effects appear to be on the severity of disease (risk of death, in-hospital case-fatality risk) rather than on the incidence of infection, as we found no effect on hospitalization risk. Developing a BCG reaction, larger reaction sizes, and positive TSTs are all associated with reduced all-cause mortality, and the main determinant of these reactions is the BCG strain administered. Since all BCG strains tested did produce some degree of BCG reactions and TST reactivity, it would be fair to assume that all are associated with some degree of beneficial NSEs.

A large-scale RCT with adequate power to test strain differences for both specific and non-specific endpoints have yet to be conducted. We have just finished enrolling neonates in a large comparison of BCG-Japan versus BCG-Russia involving 15,600 neonates and now await a further six months of follow-up before the important results can be published. A third RCT of similar size is planned.

Triangulation of the currently available data nevertheless suggests that there would likely be substantial public health advantages obtained at low-cost by:

1) Eliminating restrictive vial policies or at last adjusting wastage calculations to reflect the actual number of doses that can be retrieved from a 20-dose vial.

2) Ensuring provision of at-birth BCG vaccination administered by well-trained personnel and with monitoring of post-vaccination wheal sizes.

3) Prioritizing immunogenic strains such as BCG-Japan over BCG-Russia. BCG-Denmark produced by SSI is no longer available, and the genetically identical BCG-Denmark now produced by AJ Vaccines to our knowledge not yet been examined for its ability to induce scars, TST responses or its overall NSEs.

4) Continuously evaluating both early-life BCG vaccination coverage and the prevalence of BCG reactions, BCG reaction sizes, and TST responses to ensure high standards of vaccination programs. Such an approach could detect whether less-efficient BCG strains and/or insufficient vaccination quality are compromising program efficacy.

Dansk resumé (summary in Danish)

Denne Ph.D.-afhandling undersøgte betydningen af forskellige BCG stammer for den overordnede sygelighed og dødelighed. Vaccination ved fødslen med Bacille Calmette-Guérin er anbefalet i lande hvor TB og/eller spedalskhed er endemisk og >120 millioner børn bliver vaccineret hvert år. Mens BCG giver god beskyttelse mod de sværeste former for TB yder vaccinen kun beskyttelse i størrelsesordenen 0 til 80% overfor pulmonal TB. Der er voksende evidens fra befolkningsstudier og lodtrækningsstudier der indikerer, at den danske BCG stamme har gavnlige non-specifikke effekter (NSE) som reducerer dødeligheden fra alle årsager på 30-50%. Effekten på risikoen for hospitalsindlæggelse var ikke blevet undersøgt før denne Ph.D.-afhandling. Blandt BCG-vaccinerede børn har dem der udvikler en BCG reaktion på huden og/eller et tuberkulin respons en 30-50% lavere dødelighed end dem uden en reaktion. Karakteristika for de forskellige responstyper har ikke været undersøgt før denne Ph.D.-afhandling. BCG er en levende, svækket vaccine som produceres på en række forskellige laboratorier i verden. Vaccinen er således ikke et standardiseret eller farmakologisk veldefineret produkt. Forskellige BCG formulationer er genetisk forskellige pga. ophobning af genetiske mutationer og forskelle i laboratoriernes produktionsteknik påvirker både det absolutte antal af bakterier og ratioen mellem levende og døde bakterier i de forskellige vacciner. Det er imidlertid uvist om forskellige BCG stammer er bioekvivalente. Den globale markedsandel i 2018 for de genetisk identiske BCG-Danmark og BCG-Green Signal er 24%, BCG-Bulgarien 20%, BCG-Japan 9% og BCG-Rusland 41%. Ingen af disse større BCG stammer er nogensinde blevet evalueret i en randomiseret trial. Pga. BCGs brede anvendelse og de markante NSE har vi undersøgt effekten på overordnet dødelighed og sygelighed og på markører for beskyttelse mod TB og et velfungerende immunforsvar af at yde neonatal vaccination med forskellige BCG stammer i Guinea-Bissau. Vi havde følgende hypoteser:

Hypotese A. *BCG vacciner har gavnlige, non-specifikke effekter mht. dødelighed og sygelighed. Eftersom BCG stammer er fænotypisk forskellige er størrelsen på disse effekter*

måske også forskellige. Sammenlignet med BCG-Rusland er vaccination med BCG-Danmark og BCG-Japan associeret med færre indlæggelser, lavere dødelighed blandt indlagte og færre dødsfald.

Hypotese B. *Andelen af børn med et BCG ar og et positivt PPD respons er 30% lavere blandt børn vaccineret med BCG-Rusland sammenlignet med BCG-Danmark og BCG-Japan.*

Hypotese C. *BCG vacciner er kendt for deres sikkerhedsprofil. Eftersom BCG-Danmark og BCG-Japan formentlig er mere potente vacciner har de formentlig en større bivirkningsrate end BCG-Rusland.*

I løbet af dataindsamlingen til denne Ph.D. opstod nogle nye idéer. Fra observationer og personlige erfaringer vedr. forskellige BCG hudreaktioner besluttede den Ph.D. studerende sig for at undersøge vigtige af reaktionstypen og størrelsen af tidlige BCG hudreaktioner og effekten af disse reaktionskarakteristika på den efterfølgende overordnede dødelighed. Og pga. input fra vores vaccinatører sammenlignede vi to BCG stammer mht. indholdet af neonatale BCG doser i en vaccineflaske. BCG afsendes fra fabrikanten som en flaske indeholdende frysetørrede bakterier som skal opblandes med en flaske indeholdende 1 ml saltvand. Ifølge fabrikanterne indeholder disse flasker i alt 20 doser BCG à 0.05 ml til børn under et år og ti doser à 0.1 ml til børn over et år og voksne. Det ville dog kun være muligt at udtrække og administrere et sådant antal doser hvis der intet tab af væske forekommer i de forskellige trin, herunder både i sprøjterne anvendt til opblanding og vaccination samt i diluent- og vaccineflaskerne. Vi har undersøgt om det er muligt at udtrække 20 neonataldoser fra BCG-Rusland og BCG-Danmark (Artikel I). Vores erfarne vaccinatører kunne kun udtage 13 til 15 doser fra begge typer vaccineflasker. Dette har vigtige konsekvenser fordi vaccinespild udregnes baseret på antagelsen om at der er 20 doser i flaskerne; vaccinespildet overvurderes derfor og dette medfører uhensigtsmæssige forsinkelser i BCG vaccination forårsaget af restriktive vaccineåbningspolitikker, der er indført for at reducere vaccinespild. I artikel II har vi supplementeret de eksisterende data vedr. effekten af BCG-Danmark på dødelighed med data på effekten ift. incidensen af hospitalsindlæggelser og dødelighed blandt indlagte på tværs af tre RCTer (6.583 børn). BCG-Danmark påvirkede ikke risikoen for hospitalisering, men reducerede sygdommens sværhedsgrad, idet den neonatale BCG versus ingen-BCG CFR RR var 0.58 (0.35-0.94). Tidlig BCG reducerede særligt risikoen for fatal neonatal sepsis, for hvilket CFR RR var 0.46 (0.22-0.98). I artikel III undersøgte vi effekten af at give forskellige BCG stammer på sygelighed, dødelighed, BCG hud reaktioner, tuberkulin reaktioner og bivirkninger (ipsilateral lymfadenitis). Vi gennemførte en stor RCT som sammenlignede BCG-Danmark med BCG-Rusland og var planlagt til at inkludere 12.000 nyfødte på HNSMs fødeafdeling. Pga. et produktionsstop i 2015 på BCG produktionsenheden på Statens Serum Institut i København løb vi tør for BCG-Danmark i juli 2016, næsten midtvejs i studiet. Vi havde søgt de nødvendige tilladelser for at udskifte BCG-Danmark med BCG-Japan i resten af studiet og nåede 12.000 inklusioner i oktober 2017. BCG stammerne påvirkede hverken sygeligheden eller dødeligheden ved seksugersalderen, men studiet havde også begrænset statistisk power pga. skiftet i intervention, særligt ift. dødelighedssammenligningen. Der var dog en tendens til færre dødsfald associeret med BCG-Japan og Japan/Rusland MRR var således 0.71 (0.43-1.19). BCG-Danmark og BCG-Japan er sandsynligvis mere immunogene end BCG-

Rusland eftersom de gav flere og større BCG hudreaktioner, flere tuberkulinreaktioner, og flere bivirkninger. Disse forskelle mellem stammerne har muligvis vigtige implikationer for både den specifikke beskyttelse mod TB og for det overordnede helbred (de non-specifikke effekter). Der var indikationer af vigtige forskelle som vi er ved at evaluere i et opfølgningsstudium. Vi fandt en signifikant forskel i andelen af børn med et BCG ar, men meget mindre end hypotesen på en 30% forskel. Der var en tendens til at både BCG-Danmark og BCG-Japan var associeret med flere bivirkninger sammenlignet med BCG-Rusland, men vi manglede power for at nå definitive konklusioner. Prævalensen af BCG ar var høj for alle BCG stammerne. Andelen med et positivt TST respons til 2 TU stimulation var 65% lavere for BCG-Rusland sammenlignet med BCG-Danmark og 46% lavere for BCG-Rusland sammenlignet med BCG-Japan. Forekomsten af bivirkninger (lymfadenitis) blandt børn der blev besøgt i hjemmet var 1.0% for BCG-Danmark versus 0.2% for BCG-Rusland (p=0.12) og 1.2% for BCG-Japan versus 0.0% for BCG-Rusland (p=0.03). I Artikel IV udforskede vi ideen om at BCGs reaktionskarakteristika påvirker den overordnede dødelighed og at den givne BCG stamme er den vigtigste determinant. I en stor kohorte på 6.012 børn som havde modtaget BCG indenfor en uge efter fødslen havde 97% (5.804/6.012) en BCG hudreaktion ved to måneder og 2-12-måneders mortaliteten var 1.9% blandt børn med en hudreaktion versus 4.8% for dem der ikke havde en hudreaktion. Således var hudreaktioner efter BCG versus ingen hudreaktion associeret med en aMRR på 0.50 (0.26-0.96). Af interesse er det også at mortalitetsrisikoen var omvendt proportional med størrelsen på BCG hudreaktionerne, og risikoen var således 2.9% for børn med små reaktioner, 1.8% for medium reaktioner og 0.8% for store reaktioner. Den tilsvarende stor versus lille reaktion aMRR var således 0.35 (0.20-0.63) og der var en lineær trend af faldende dødelighed for stigende reaktionsstørrelse (p for trend<0.001). Vi konkluderede at den vigtigste determinant både for at udvikle en BCG hudreaktion og størrelsen på reaktionen var den anvendte BCG stamme; BCG-Danmark og BCG-Japan var associeret med flere og større reaktioner end BCG-Rusland. I Artikel V evaluerede vi effekten af at have en TST reaktion (defineret som >1mm) ved to- og seks måneder versus ingen reaktion på dødeligheden fra alle årsager op til 12 måneder. Alle børn i kohorte havde modtaget BCG i løbet af neonatalperioden. At have en TST reaktion ved to måneder versus ingen reaktion var associeret med en 2-12-måneders aMRR på 0.34 (0.12-0.94). Ved seks måneder var aMRR 0.66 (0.15-2.92). Vi gennemførte en meta-analyse af de tilgængelige studier og på tværs af fire kohorter fra Guinea-Bissau da var en positiv tuberkulin reaktion versus ingen reaktion associeret med en aMRR på 0.56 (0.38-0.83). Tilsvarende var effekten på dødeligheden af at have en reaktion ved 6 måneder versus ingen reaktion en aMRR på 0.63 (0.39-1.00) på tværs af fire kohorter. De vigtigste determinanter for at udvikle en tuberkulinreaktion ved seks måneder var størrelsen af post-vaccinationspaplen (at modtage en stor dosis BCG) og at blive vaccineret med BCG-Danmark eller BCG-Japan og ikke BCG-Rusland. Som konklusion støtter vores fund at BCG har afgørende NSE som øger overlevelsen markant. Disse effekter er tilsyneladende i højere grad på sygdommens *sværhedsgrad* (risiko for død, risiko for død blandt indlagte) end på incidensen for infektion, eftersom vi ikke fandt en effekt på hospitaliseringsrisikoen. Både at udvikle en BCG reaktion, større reaktionsstørrelse og positive TSTer var associeret med reduceret dødelighed fra alle årsager, og den vigtigste determinant for disse reaktioner var den administrerede BCG

stamme. Eftersom alle BCG stammer udløste en vis grad af BCG reaktioner og TST reaktivitet må det være rimeligt at formode at de alle er associeret med en vis grad af gavnlige NSE. En RCT i større skala med tilstrækkelig power til at teste forskelle mellem stammer for både specifikke og non-specifikke endepunkter mangler stadig at blive gennemført. Triangulering af de tilgængelige data tyder imidlertid på at der ville være markante fordele for folkesundheden med få omkostninger ved at:

1) Eliminere restriktive vaccine åbningspolitikker, eller i hvert fald justere beregninger af vaccinespild således at de afspejler det reelle indhold af doser i en 20-dosis flaske.

2) At sikre distribution af BCG-ved-fødslen administreret af veluddannet personale og med monitorering af post-vaccinations papelstørrelser.

3) Prioritering af immunogene stammer såsom BCG-Japan over BCG-Rusland. BCG-Denmark produceret af SSI er ikke længere tilgængelig og den genetisk identiske BCG-Danmark som nu produceres af AJ Vaccines er iflg. vores information ikke blevet undersøgt endnu for sin evne til at inducere BCG ar, TST responser eller non-specifikke effekter.

4) Løbende evaluering af vaccinationsdækningen med BCG, prævalensen af BCG reaktioner, reaktionsstørrelser og TST responser mhp. at sikre høje standarder i vaccinationsprogrammerne. En sådan fremgangsmåde ville kunne detektere om mindre effektive BCG stammer og/eller lav vaccinationskvalitet kompromitterer programmets effektivitet.

Resumo em português (summary in Portuguese)

Esta dissertação de Doutorado (Ph.D.) examinou a importância das diferentes cepas de BCG relativamente à morbidez e à mortalidade global. A vacinação por ocasião do nascimento com Bacille Calmette-Guérin é recomandada nos países nos quais a TB e/ou a lepra são endémicas e >120 milhões de crianças são vacinadas anualmente. Ao passo que a BCG proporciona uma boa protecção contra as formas mais severas de TB, a vacina apenas dá uma protecção na ordem de 0 a 80% contra a TB pulmonar. Há uma evidência cada vez maior de estudos de populações e estudos randomizados que indica que a cepa dinamarquesa de BCG tem efeitos não-específicos (ENE) benéficos, que reduzem a mortalidade por todas as causas em 30% a 50%. O efeito sobre o risco de hospitalização não havia sido examinado antes desta dissertação de Doutorado. Dentre as crianças vacinadas com BCG, as que desenvolvem uma reacção à BCG na pele e/ou uma resposta de tuberculina têm uma mortalidade 30% a 50% inferior que as que não desenvolvem qualquer reacção. As características dos diferentes tipos de resposta não haviam sido examinadas antes desta dissertação de Doutorado. A BCG é uma vacina viva enfraquecida que é produzida numa série de diferentes laboratórios ao redor do mundo. Assim sendo, a vacina não é um produto padronizado ou farmacologicamente bem definido. As diferentes formulações de BCG são geneticamente diferentes devido ao acúmulo de mutações genéticas, e as diferenças nas técnicas de produção dos laboratórios influenciam tanto o número absoluto de bactérias como a proporção entre bactérias vivas e mortas nas diferentes vacinas. No entanto, não se sabe se as diferentes cepas de BCG são bio-equivalentes. A participação de mercado global em 2018 do BCG-Dinamarca e do BCG-Green Signal foi de 24%, BCG-Bulgária 20%, BCG-Japão 9% e BCG-Rússia 41%. Nenhuma

dessas principais cepas de BCG foi avaliada entre si em um estudo randomizado.Devido à ampla utilização da vacina BCG e os ENEs marcantes, examinámos, na Guiné-Bissau, o efeito da vacinação neonatal com diferentes cepas de BCG sobre a mortalidade e a morbidez global, sobre os marcadores de protecção contra a TB e sobre a obtenção de uma defesa imunológica optimizada. Tínhamos as seguintes hipóteses:

Hipótese A. *As vacinas BCG têm efeitos não-específicos benéficos sobre a mortalidade e a morbidez. Dado que as cepas de BCG são fenotipicamente diferentes, a amplitude destes efeitos também deveriam ser diferentes. Em comparação com a BCG-Rússia, a BCG-Dinamarca e a BCG-Japão são associadas com menos hospitalizações, mortalidade mais baixa entre os hospitalizados e menos óbitos.*

Hipótese B. *A quota-parte de crianças com uma cicatriz de BCG e uma resposta PPD positiva é 30% mais baixa dentre as crianças vacinadas com BCG-Rússia em comparação com a BCG-Dinamarca e a BCG-Japão.*

Hipótese C. *As vacinas BCG são conhecidas por seu perfil de segurança. Dado que a BCG-Dinamarca e a BCG-Japão provavelmente são vacinas mais potentes, é bem possível terem uma taxa de efeitos colaterais maior que a BCG-Rússia.*

Durante a colecta dos dados para esta dissertação, surgiram novas ideias. A partir de observações e experiência pessoal, o doutorando decidiu examinar a importância do tipo de reacção e o tamanho das reacções precoces da BCG na pele e o efeito dessas características de reacção sobre a mortalidade geral subsequente. E devido ao subsídio de nossos vacinadores, comparámos duas cepas de BCG relativamente ao contéudo de doses de BCG neonatais num frasco de vacina. A BCG é remetida pelo fabricante na forma de um frasco contendo bactérias secas por liofilização que devem ser diluídas com o conteúdo de um frasco contendo 1 ml de salmoura. Segundo os fabricantes, estes frascos contém um total de 20 doses de BCG de 0,05 ml para crianças menores de um ano e doses de 0,1 para crianças maiores de um ano, assim como adultos. Porém, só seria possível extrair e administrar este número de doses, se não houvesse qualquer perda de líquidos nas diferentes etapas, nomeadamente nas seringas utilizadas para a diluição e vacinação, bem como nos frascos de diluente e vacina. Examinámos se é possível extrair 20 doses neonatais da BCG-Rússia e da BCG-Dinamarca (Artigo I). Os nossos vacinadores experientes apenas puderam extrair 13 a 15 doses de ambos os tipos de frasco de vacina. Isso tem consequências importantes, porque o desperdício de vacina é calculado com base na presunção de que há 20 doses nos frascos; portanto, o desperdício de vacina é superestimado e isso implica atrasos inconvenientes na vacinação de BCG causados por políticas restritivas de abertura dos frascos de vacina, introduzidas para reduzir o desperdício de vacina. No Artigo II, complementámos os dados existentes sobre o efeito da BCG-Dinamarca relativamente à mortalidade dentre os hospitalizados com dados sobre a incidência das hospitalizações e da mortalidade dentre os hospitalizados através de três RCTs (6.583 crianças). A BCG-Dinamarca não influenciou o risco de hospitalização, mas reduziu a gravidade da doença, visto que o CFR RR da BCG neonatal versus nenhuma BCG foi

de 0,58 (0,35-0,94). A BCG precoce reduziu, em especial, o risco de sepse neonatal fatal, para a qual o CFR RR foi de 0,46 (0,22-0,98). No Artigo III, examinámos o efeito de ministrar diferentes cepas de BCG sobre a morbidez, mortalidade, reacções à BCG na pele, reacções de tuberculina e efeitos colaterais (linfadenite ipsilateral). Realizámos um grande RCT que comparou a BCG-Dinamarca com a BCG-Rússia e tínhamos planeado incluir 12.000 recém-nascidos na maternidade do HNSM. Devido a uma desactivação da produção, em 2015, na unidade de produção de BCG do Statens Serum Institut em Copenhaga, a BCG-Dinamarca esgotou em Julho de 2016, quase no meio do estudo. Havíamos solicitado as autorizações necessárias para substituir a BCG-Dinamarca com a BCG-Japão no resto do estudo e chegámos a 12.000 inclusões em Outubro de 2017. As cepas de BCG não influenciaram a morbidez ou a mortalidade às seis semanas de idade, porém o estudo também teve poder (power) estatístico limitado devido à mudança de intervenção, em especial, relativamente à comparação da mortalidade. No entanto, houve uma tendência de menos óbitos associados com a BCG-Japão e a aMRR Japão/Rússia foi portanto de 0,71 (0,43-1,19). A BCG-Dinamarca e a BCG-Japão são, provavelmente, mais imunógenos que a BCG-Rússia, já que resultaram em mais e maiores reacções de BCG na pele, mais reacções de tuberculina e mais efeitos colaterais. Essas diferenças entre as cepas têm, provavelmente, implicações importantes, tanto para a protecção específica contra TB, como para a saúde em geral (os efeitos não-específicos). Houve indicações de diferenças importantes que estamos a avaliar num estudo de acompanhamento. Encontrámos uma diferença significante no número de crianças com cicatrizes de BCG, porém muito menos do que a hipótese sugerida de uma diferença de 30%. Havia uma tendência de, tanto a BCG-Dinamarca, como a BCG-Japão estarem associadas a mais efeitos colaterais em comparação com a BCG-Rússia, mas nos faltou poder estatístico para chegar a conclusões definitivas. A prevalência de cicatrizes de BCG era alta para todas as cepas de BCG. A quota-parte com uma resposta de TST positiva para o estímulo 2 TU foi 65% mais baixa para a BCG-Rússia em comparação com a BCG-Dinamarca, e 46% mais baixa para a BCG-Rússia em comparação com a BCG-Japão. Os efeitos colaterais (linfadenite) entre as crianças visitadas em casa foi de 1% para a BCG-Dinamarca versus 0,2% para a BCG-Rússia (p=0,03). No Artigo IV, examinámos a ideia que as características de reacções da BCG influenciarem a mortalidade global e que a respectiva cepa de BCG era o factor determinante mais importante. Numa grande coorte de 6.012 crianças que haviam recebido BCG dentro de uma semana após o nascimento, 97% (5.804/6.012) tinham uma reacção à BCG na pele na idade de dois meses, e entre os 2 e 12 meses a mortalidade era de 1,9% dentre as crianças com uma reacção à BCG na pele versus 4,8% para as crianças sem reacção na pele. Assim, as reacções de BCG na pele versus nenhuma reacção na pele estiveram associadas a uma aMRR de 0,50 (0,26-0,96). Interessante foi também o facto de que o risco de mortalidade era inversamente proporcional ao tamanho das reacções à BCG na pele, sendo o risco de 2,9% para as crianças com pequenas reacções, de 1,8% para as reacções médias e de 0,8% para as grandes reacções. A aMRR correspondente da reacção pequena versus a grande era, portanto, de 0,35 (0,20-0,63) e havia uma tendência linear de mortalidade em queda proporcional ao aumento do tamanho da reacção (p para tendência>0.001). Concluímos que o determinante mais importante, tanto para desenvolver uma reacção à BCG na pele, como para o tamanho da reacção era a cepa de BCG utilizada; a BCG-Dinamarca e a BCG-Japão estavam associadas a mais e maiores reacções do que a BCG-

Rússia. No Artigo V, avaliámos o efeito de ter uma reação à tuberculina (TST, definida como >1mm) aos dois e seis meses versus não ter nenhuma reacção, relativamente à mortalidade por várias causas até aos 12 meses. Todas as crianças na coorte tinham recebido BCG durante o período neonatal. Ter uma reação de TST aos dois meses versus não ter nenhuma reacção estava associado a uma aMRR entre os 2 e 12 meses de 0,34 (0,12-0,94). Aos seis meses, a aMRR era de 0,66 (0,15-2,92). Realizámos uma metanálise dos estudos disponíveis e, através de quatro coortes na Guiné-Bissau, houve uma reacção positiva de tuberculina versus nenhuma reacção associada a uma aMRR de 0,56 (0,38-0,83). Correspondentemente, o efeito sobre a mortalidade de ter uma reacção aos seis meses versus nenhuma reacção era uma aMRR de 0,63 (0,39-1,00) através de quatro coortes. Os principais determinantes para desenvolver uma reacção de tuberculina aos seis meses era o tamanho da papula pós-vacinação (receber uma grande dose de BCG) ou ser vacinado com BCG-Dinamarca ou BCG-Japão, mas não BCG-Rússia. Para concluir, o nosso achado sustenta que a BCG tem ENEs determinantes, que aumentam marcadamente a sobrevida. Aparentemente, esses efeitos são mais fortes sobre a *gravidade* da doença (risco de morrer, risco de morte entre os hospitalizados) do que sobre a incidência da infecção, já que não encontrámos qualquer efeito sobre o risco de hospitalização. Tanto o desenvolvimento de uma reacção à BCG, como ter uma maior reacção e TSTs positivos estava associado a uma mortalidade reduzida por todas as causas, e o determinante mais importante de todas essas reacções era a cepa de BCG ministrada. Já que todas as cepas de BCG causaram um certo grau de reacções à BCG e reactividade ao TST, deve ser razoável presumir que todas estão associadas a um certo grau de ENEs benéficos. Um RCT em grande escala com poder suficiente para testar as diferenças entre as cepas quanto a fins específicos e não-específicos ainda falta ser realizado. No entanto, a triangulação dos dados disponíveis sugere que pode haver benefícios marcantes para a saúde da população, a baixos custos, em:

1) Eliminar as políticas restritivas de abertura de frascos de vacina ou, pelo menos, reajustar os cálculos de desperdícios de vacina, de forma que reflitam o conteúdo real de doses num frasco de 20 doses.

2) Assegurar a distribuição da vacina BCG por ocasião do parto, ministrada por um pessoal bem formado e com monitoração do tamanho pós-vacina das papulas.

3) Priorização das cepas imunógenas, tais como a BCG-Japão em lugar da BCG-Rússia. A BCG-Dinamarca, produzida pelo Statens Serum Institut (Instituto Pasteur da Dinamarca), não está mais disponível e a BCG-Dinamarca, geneticamente idêntica, produzida pela AJ Vaccines, segundo as nossas informações ainda não foi examinada pela capacidade de indução de cicatrizes de BCG, respostas TST ou efeitos não-específicos.

4) Uma avaliação corrente da cobertura da vacinação com BCG, da prevalência de reacções à BCG, dos tamanhos das reacções e das respostas TST, visando a assegurar altos padrões nos programas de vacinação. Um tal procedimento poderia detectar se as cepas de BCG menos eficazes e/ou uma baixa qualidade de vacinação comprometem a eficácia do programa.

1 Introduction

BCG vaccination at birth is recommended in TB-endemic areas to contain *Mycobacterium tuberculosis,* and in lepra-endemic areas to contain *Mycobacterium lepra*.[1] BCG is effective against the more severe forms of TB, such as miliary and cerebral TB, and provides moderate protection against pulmonary TB.[2] Interestingly, it has been discovered that BCG also affects the severity of untargeted infections, lowering the all-cause mortality in areas with high infectious disease pressure.[3] Observational studies from Guinea-Bissau have indicated that there are substantial beneficial effects on all-cause mortality among BCG-vaccinated infants for those that produce a BCG scar[4] or a positive tuberculin reaction versus those without such reactions.[5–7] In a series of RCTs comparing early-BCG-Denmark versus no BCG conducted in Guinea-Bissau among LBW infants, those that received early-BCG-Denmark had a substantially lower neonatal mortality risk compared to those that were not vaccinated.[8] These marked beneficial effects are not explained by protection against TB, which is rare in infants. The beneficial effects of BCG vaccination appear most pronounced during the first months of life, during which the overall mortality risk is higher and other vaccines have not yet been administered. BCG is a strong immunostimulant, and its beneficial effects on the immune system are thought to be mediated by epigenetic modifications of innate immune cells. This concept is known as *trained innate immunity*.[9] A study in mice has demonstrated that access of BCG to the bone marrow changes the transcriptional landscape of hematopoietic stem cells and multipotent progenitors, inducing immune training.[10] A recent analysis in humans demonstrated that BCG induces transcriptomic myeloid priming of the hematopoietic stem and progenitor cell compartment, upregulating myeloid and granulocytic pathways and inducing transcription factors connected to myeloid cell function.[11] This resulted in elevated granulocyte numbers in BCG-vaccinated infants and induced lasting innate immune system memory.

In 2014, the WHO commissioned the Strategic Advisory Group of Experts on Immunization (SAGE) to review the evidence of NSEs of several childhood vaccines. SAGE concluded that "*Estimated effects (of BCG) are in the region of a halving of mortality risk (...)"* and recommended further studies into these effects.[12]

Surprisingly, BCG vaccines are not a pharmacologically well-defined product.[13] BCG strains are live-attenuated mycobacteria that have accumulated substantial genetic diversity from the inception of BCG almost a century ago to the introduction of freeze-dried seed-lots in the 1960s.[14] Aside from the genetic differences, the specific laboratory techniques applied at a given manufacturing unit can also alter the properties of a specific BCG strain. This was seen when a substantial increase in adverse events (BCG osteitis) after vaccination with BCG-Gothenburg was noted in Finland, coinciding with the 1971 transfer of the BCG-Gothenburg production from Sweden to Statens Serum Institut in Denmark.[15]

Only a few studies have evaluated the effects of different BCG strains and some of the strains that were previously tested in RCTs are now obsolete.[16–19] A substantial

difference in protective efficacy against TB was noted in an RCT from Hong Kong, but the full trial results were never published.[16] No trial has evaluated NSEs between different strains as the main outcome and the strains currently prequalified by WHO were never tested against each other in a large-scale RCT before the present Ph.D. project.

Observational studies have indicated that BCG-Japan and BCG-Denmark are more immunogenic than BCG-Russia.[20,21] These findings were corroborated by a small RCT conducted in Australia. The trial reported a higher proportion of polyfunctional CD4 T-cells and higher concentrations of secreted Th1 cytokines associated with BCG-Denmark and BCG-Japan when compared to BCG-Russia.[21] The study also reported a higher BCG reaction prevalence and larger BCG reactions associated with BCG-Denmark and BCG-Japan when compared to BCG-Russia. A retrospective observational study from Kazakhstan has indicated superior protection against TB associated with BCG-Japan when compared to BCG-Russia.[22]

At the Maternity Ward of the National Hospital in Guinea-Bissau, the BHP has conducted a series of RCTs evaluating health interventions provided at birth without interruptions since 2002. This platform and the established Health Demographic Surveillance System enabled us to conduct a large-scale RCT evaluating BCG strains for their effects on morbidity, mortality, BCG skin reactions, tuberculin reactivity, and adverse events.

Aside from the possible differences in specific and non-specific effects associated with different BCG strains, there are currently approximately eighteen novel TB-vaccine candidates under development.[23] Many of these are either sub-unit/booster vaccines designed to supplement BCG vaccination at birth, vaccines using BCG as a vector to deliver additional vaccine antigens, or recombinant BCG strains designed to overexpress certain genes associated with immunogenicity.[24] With BCG thus often being the building block for novel vaccine candidates, the characterization and understanding of the diverse strains are important. A given TB-vaccine candidate will also likely face Phase III testing against a strain of BCG to demonstrate safety, immunogenicity, and protection against TB. It is thus essential to establish whether there are clinically relevant differences between different BCG strains and, if possible, which strain is the most efficient and beneficial for both specific and non-specific outcomes. If clinically relevant differences exist and clinicians and policymakers take action, the result would be better protection against TB and reductions in all-cause mortality. Ideally, no novel TB-vaccine should end up replacing BCG because of having been tested against a weak BCG strain or because NSEs were not taken into consideration. Given that >120 million infants are BCG-vaccinated every year[13], the public health implications of BCG vaccination are substantial.

2 Background

2.1 The inception of the first BCG strain

Tuberculosis has been a ubiquitous human companion for thousands of years, as evidenced by the presence of *M. tuberculosis* DNA in Egyptian mummies buried in the pyramids.[25] The historical impact on societies has been tremendous and so is the impact today. TB is one of the top 10 causes of death worldwide and the leading cause of death from a single infectious agent (ranking above HIV/AIDS), claiming 1.5 million lives in 2018.[26] After the first vaccine against smallpox was successfully introduced by Edward Jenner in 1796, the hunt for a vaccine against TB was intensified. The smallpox vaccine was derived from the cow (Latin: *vacca)* after the serendipitous discovery that milkmaids exposed to cowpox were protected against smallpox, coining the term for this novel concept, *vaccines*. Finding a vaccine that could protect against TB without being able to cause TB in the vaccinated individual was a challenging exercise. A virulent bovine strain of *m. tuberculosis* isolated from the udder of a TB-infected cow (*Lait Nocard)* in 1908 thus had to be maintained in a potato-bile medium through repeated growth cycles for 13 years.[27] This work was carried out by the two bacteriologists and immunologists Calmette and Guérin. There were great grievances securing the growth mediums through World War I and extensive testing of vaccine safety was performed in a range of different laboratory animals. Finally, oral BCG was administered to humans for the first time in Paris in 1921 without indications of major adverse events in the recipients.[27] By 1924, a report of 660 oral BCG vaccinations to infants was published and there had been no serious adverse events.[28] Given the disease-burden and mortality associated with TB there was high demand for BCG. This led to the rapid dissemination of the early strain maintained at the Pasteur Institute, long before uniform standards in terms of culturing protocols and seed lots had been established.[29] BCG-Pasteur vaccine strains thus reached Russia in 1921, Brazil and Japan in 1924, Sweden in 1926 and Denmark in 1931 (Figure 1).[30]

Figure 1. Three vials of BCG-Pasteur with expiry date March 18, 1931. Photo Credit: Alfred Eisenstaedt/AP/Shutterstock.

When appropriate genetic sequencing techniques had been developed, it was subsequently shown that significant mutations had already accumulated at the Pasteur Institute within

the few years that separated the shipments of the different strains.[31] The strains continued to be cultivated in the respective laboratories applying slightly different laboratory techniques. During this process, the strains accumulated additional genetic mutations (deletions, duplications and single nucleotide polymorphisms) until lyophilization with the establishment of BCG seed-lots for the different strains was introduced by the WHO in the 1960s.[32] BCG has thus been administered in its different strain formulations since 1921 and is now approaching its 100th anniversary. It is estimated that >4 billion humans have been vaccinated, and yearly vaccinations amount to >120 million per year.[31] Despite such extensive use and research regarding BCG, there is still plenty to be understood about BCG, and a recent editorial[33] pointed to three important questions that remain unanswered:

1) What is BCG's mechanism of action?

2) Does BCG provide non-specific protection against infectious and/or non-infectious diseases, beyond TB?

3) What are the consequences of the evolution of BCG (and the associated pool of BCG strains)? Are BCG strains bioequivalent?

2.2 BCG strains prequalified by WHO for use by UNICEF

The BCG vaccines currently prequalified by the WHO for use by UNICEF are the genetically identical[34] BCG-Bulgaria & BCG-Russia, along with BCG-Japan and the two genetically identical strains BCG-Denmark & BCG-Green Signal (Figure 2).[35]

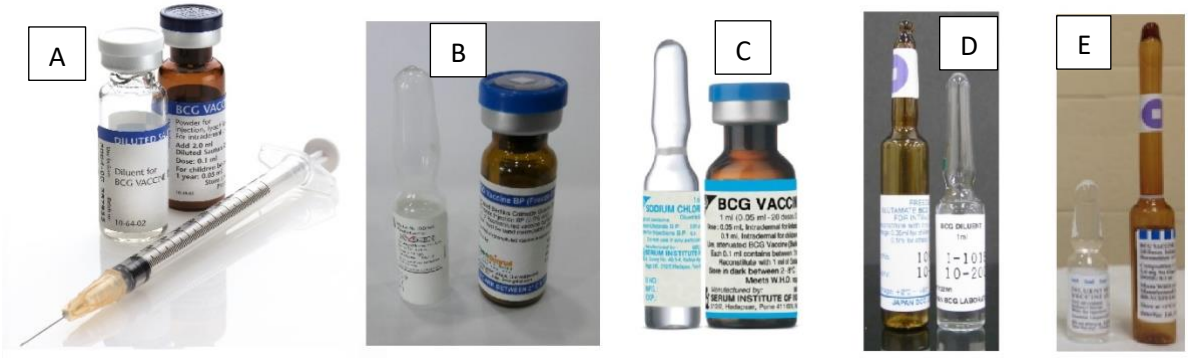


Figure 2. WHO prequalified BCG strain product presentations from producers. Note: A: BCG-Denmark (Photo credit: WHO). B: BCG-GreenSignal (Photo credit: GreenSignal Bio Pharma ltd). C: BCG-Russia (Photo credit: WHO). D: BCG-Japan (Photo credit: WHO). E: BCG-Bulgaria (private photo).

WHO prequalification is a system that aims to ensure that vaccines used in immunization programs are safe and effective. The system encompasses a review of the production process and quality control procedures, along with laboratory testing and audits of manufacturing facilities.[36] Prequalified BCG strains are considered equal, and are the most widely used in the world since most countries procure vaccines via the UNICEF route. The producers of prequalified BCG strains reach 169 countries, whereas an additional fifteen producers of non-prequalified BCG strains can serve 52 countries where their product is registered.[37] In 2013, the BCG vaccine strain quantities delivered to the UNICEF supply division were: BCG-Bulgaria (44 million doses), BCG-Japan (34 million doses), BCG-Russia (38

million doses) and BCG-Denmark (1 million doses).[38] For 2018, the vaccine purchase data for the major BCG strains are summarized in Table 1.

Table 1. Overview of 2018 vaccine purchase data for major BCG strains, in million doses.[a]

Manufacturer	Strain	Self-procurement	UNICEF procurement	Total	Market share
AJ Vaccines	Denmark	2.2	0	2.2	1%
Green Signal Bio Pharma Limited	Green Signal	22.7	20.4	43.1	23%
Intervax BB-NCIPD Ltd.	Bulgaria	0	37.6	37.6	20%
Japan BCG Laboratory	Japan	2.8	13.8	16.6	9%
Serum Institute of India Pvt. Ltd.	Russia	22.8	53.7	76.5	41%
Microgen	Russia	8.5	0	8.5	5%
Total		59	125.5	184.5	100%

[a]Data extracted October 18, 2019, from the WHO MI4A/V3P Vaccine Purchase Database.[39] Countries that are self-supplying with BCG such as Brazil (BCG-Moreau), China (BCG-China) and Vietnam (BCG-Vietnam) do not procure vaccines via these channels. With approx. 14 million births occurring per year in China[40] and the possibility that BCG-China (genetically identical to BCG-Denmark) is exported to some neighboring countries, BCG-China is likely an important BCG strain.

2.3 The TB disease-burden

The German microbiologist Robert Koch claimed that BCG accounted for one in seven deaths when he first described the bacterial etiology of TB on 24 March 1882, an event since commemorated every year as World TB Day.[41] The bacteria is no longer omnipresent in Germany and other high-income countries due to improvements in income, housing, nutrition, and the introduction of effective anti-TB drug therapies and BCG vaccination.[24,42] However TB remains an endemic disease in low-income countries and is as mentioned above the most deadly infectious agent.[42] An estimated 1.7 billion humans are infected with TB as carriers of the mycobacterium and of these, 5-10% will develop TB disease during their lifetime, corresponding to around 10 million (9-11 million) new cases per year and 1.6 million (1.5-1.7 million) deaths.[42] Miliary TB is manifested in around 1% of all reported cases of TB.[43] Similarly, it has been suggested that 100,000 individuals might develop cerebral TB per year, e.g., approx. 1% of TB cases, but the actual figure might be higher since many cases of cerebral TB remain undiagnosed.[44]

2.4 Efficacy of different BCG strains against TB

Despite the widespread use of BCG vaccination to contain TB, the protective efficacy against pulmonary TB is modest, with an estimated effect between 0-80% in different trials.[24] It has been suggested that the distance to the equator influences the efficacy of BCG since the efficacy tends to be higher in trials conducted longer from the equator, and lower in tropical and subtropical regions closer to the equator, which coincides with the regions where TB is most prevalent.[2] BCG-induced protection against TB is enhanced when vaccination is provided during infancy or at school age, provided that stringent tuberculin testing before vaccination was performed.[2] The higher presence of environmental mycobacteria in regions closer to the equator has been suggested to be the culprit of the reduced efficacy. This could either be by masking the effect of BCG (inducing protection that BCG cannot

further enhance) or by blocking its effect, for example by preventing multiplication of BCG in the host.[45]

Vaccination at birth or shortly after birth offers better protection against TB, likely because pre-vaccination exposure to environmental mycobacteria is reduced or eliminated. BCG vaccination during infancy has also consistently been associated with high estimates of efficacy against severe progressive TB disease such as miliary and cerebral TB.[46] Despite the relatively weak protection against pulmonary TB, and that widespread BCG vaccination has failed to contain the TB epidemic, BCG thus remains a highly cost-effective intervention against severe childhood TB. The Numbers-Needed-to-Vaccinate to prevent one case of cerebral or miliary TB has been estimated to 3,435 and 9,314, respectively, and BCG vaccination provides one year of healthy life gained at the cost of US$206 (150$-272$).[47] Aside from the distance to the equator and the influence of environmental mycobacteria, the varying efficacy of BCG has been speculated to be caused by different trial designs, discrepancies in the age at which vaccination was provided, and the use of different BCG strains. A large meta-analysis did not find evidence of different protective efficacy between strains, but the strains were also grouped together according to major genetic lineages, which meant that strains such as BCG-Japan and BCG-Russia were grouped and analyzed together.[2] Also, as discussed by Jayaraman et al.[48], the meta-analysis was an ecologic analysis prone to confounding, in which only two of eigtheen included trials had reported comparisons by strain. Neither reported effects of neonatal vaccination - and the two trials did not evaluate BCG-Russia and BCG-Japan. While some studies have provided indications of varying efficacy associated with different BCG strains, the question as to whether the currently used BCG strains provide different protective efficacy against TB or in their NSEs remains hitherto unanswered due to the paucity of available RCTs. As such, none of the major BCG strains currently distributed by UNICEF for use in low- and middle-income countries have been evaluated against each other in a randomized trial, despite that it has been suggested that the different BCG strains should perhaps be characterized as different vaccines.[14] The major RCTs and observational studies that have compared different BCG strains for their protective efficacy against TB are listed in Table 2.

Table 2. Major RCTs and observational studies that have compared different BCG strains for their protective efficacy against TB.

RCT	Strains compared (n)	Vaccine efficacy (VE) or relative risk (RR)	Main conclusions
Aronson, Alaska, United States, 2004[17]	BCG-Phipps (1,005)	VE 44% (95% CI -3% to 70%)	Adjusting for BCG strain and BCG dose did not substantially change the vaccine effect. Combined VE of 52% (27% to 69%), which persisted for 50-60 years.
	BCG-Pasteur (478)	VE 59% (95% CI 25% to 78%)	
	Control (1,309)	-	
Medical Research Council, The	BCG-Denmark (14,100)	VE 83% (99% CI 71% to 90%)	All participants were stringently tuberculin-tested before inclusion, and both vaccines were protective against TB.
	Control (16,000)	-	

United Kingdom, 1959[18]	Vole bacillus vaccine (6,700)	VE 87% (99% CI 73% to 96%)	BCG dose was correlated with subsequent tuberculin reactivity, but weak batches also conferred protection. There were fewer non-TB deaths among vaccinated (either vaccine) versus controls.
	Control (6,500)	-	
Tuberculosis Research Centre (ICMR), Chennai, India, 2006, tuberculin-negative subjects[19]	Low dose (0.01 mg) BCG-Denmark or BCG-Pasteur (13,315)	RR 0.77 (0.51-1.15) VE 23%	BCG was of little value in preventing sputum-positive cases of pulmonary TB in South India, but a substantial share of participants were tuberculin positive at the onset of the trial. In a subset of 40,342 that were tuberculin-negative at inclusion (presented here), a low overall level of protection, 32% (3%-52%) (29% for BCG-Denmark and 34% for BCG-Pasteur) was observed. Protection was higher (and significant) in the group that received the higher dose of BCG.
	High dose (0.1 mg) BCG-Denmark or BCG-Pasteur (13,781)	RR 0.60 (0.39-0.92) VE 40%	
	Control (13,246)	-	
Hong Kong, 1978/1982-1991[16]	BCG-Glaxo (151,425)	TB incidence 6.1/10,000	BCG-Pasteur was associated with a 45% (22%-61%) lower risk of TB than BCG-Glaxo. The full results of the trial have unfortunately never been published.
	BCG-Pasteur (151,667)	TB incidence 3.4/10,000	
Kazakhstan National Tuberculosis Center, Kazakhstan, 2002-2006[22]	BCG-Japan (168,664)	Clinical TB incidence 0.6/1,000. Culture positive TB risk: RR 0.08 (0.01-0.61) VE 92%	Observational study reporting a natural experiment. All three BCG vaccines provided protection against TB, including TB meningitis, but BCG-Japan appeared to provide superior protection against both clinical TB notifications and culture-confirmed TB.
	BCG-Serbia (150,938)	Clinical TB incidence 1.1/1,000. Culture positive TB risk: RR 0.18 (0.04-0.79) VE 82%	
	BCG-Russia (138,059)	Clinical TB incidence 1.5/1,000. Culture positive TB risk: RR 0.49 (0.17-1.38) VE 51%	
	Unvaccinated (160,970)	Clinical TB incidence 1.9/1,000.	

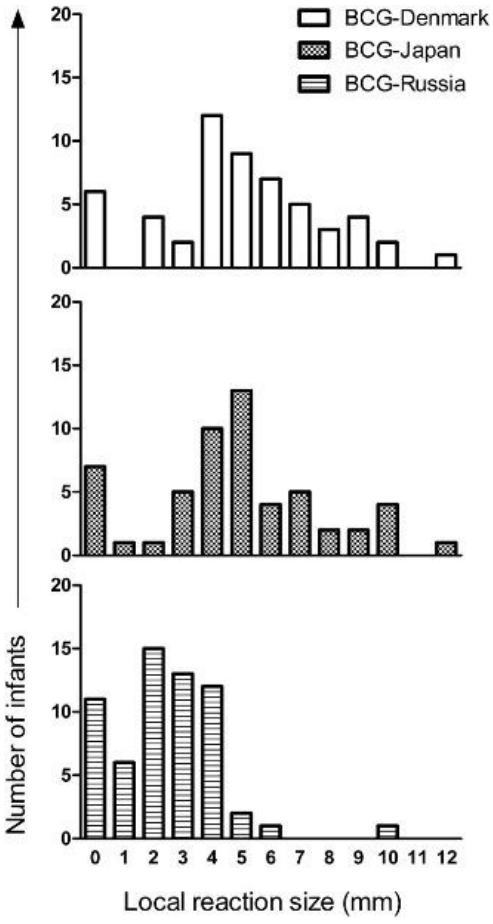
A large RCT among 303,092 neonates in Hong Kong reported that the risk of TB was 45% (22% to 61%) lower among infants vaccinated with BCG-Pasteur when compared to BCG-Glaxo.[16] In a large RCT conducted in South India, participants that were tuberculin-negative at enrollment and received a high dose of BCG-Denmark or BCG-Pasteur had a reduced risk of TB compared to unvaccinated controls.[19] An RCT from Alaska involving children and adults vaccinated between 1935-1938 with 50 years of follow-up demonstrated BCG-induced long-term protection against TB, the protective vaccine efficacy being 52% (27% to 69%).[17] Two strains of BCG were used (BCG-Phipps and BCG-Pasteur) and there was no difference between them. In a retrospective observational study from Kazakhstan, at-birth vaccination in comparable 7-month time-frames with BCG-Japan, BCG-Serbia and BCG-Russia was compared to a group of unvaccinated infants. The vaccine efficacy was compared after three years of follow-up and unvaccinated infants had not received BCG because vaccination had been temporarily suspended due to a spike in adverse event occurrence associated with BCG-Serbia.[22] All BCG strains provided some degree of protection against radiologically confirmed TB when compared to unvaccinated

infants and for culture-positive TB cases, the BCG-Russia versus unvaccinated RR was 0.49 (0.17-1.38) while the same estimate was 0.08 (0.01-0.61) for BCG-Japan.[22] All BCG strains were protective against TB meningitis.

In summary, several studies have indicated that there are possible differences between BCG strains on TB-specific outcomes. Still, no definitive conclusions have been drawn regarding differences in vaccine efficacy associated with specific BCG strains.

2.5 Specific and non-specific immune responses to different strains of BCG

Studies comparing the immunogenicity of BCG strains can provide insights into possible differences in protection against TB and NSEs associated with different strains. Such studies will often rely on surrogate outcomes, however, and while these may be correlated with beneficial effects (specific and non-specific), the clinical significance is often unclear. As such, a small RCT among 209 infants comparing BCG-Denmark, BCG-Japan, and BCG-Russia has been conducted in Australia.[21] The trial demonstrated that BCG-Denmark and BCG-Japan were associated with higher frequencies of mycobacterial-specific polyfunctional and cytotoxic T cells and higher concentrations of Th1 cytokines when compared with BCG-Russia.[21] Interestingly, the authors also reported that BCG-Denmark and BCG-Japan were associated with larger local skin reactions at the BCG injection site ten weeks after immunization, when compared to BCG-Russia ($p<0.001$). The median local reaction size for infants immunized with BCG-Denmark was 5 mm (IQR, 4-7 mm), 5 mm (IQR, 3-7 mm) for BCG-Japan, and 2 mm (IQR, 1-4 mm) for BCG-Russia (Figure 3).



Source: Ritz N, Dutta B, Donath S et al: The influence of bacille Calmette-Guerin vaccine strain on the immune response against tuberculosis: a randomized trial. Am J Respir Crit Care Med. 2012 Jan 15;185(2):213-22. Reprinted with permission of the American Thoracic Society. Copyright © 2019 American Thoracic Society. *The American Journal of Respiratory and Critical Care Medicine* is an official journal of the American Thoracic Society.

Figure 3. Distribution of BCG scar sizes by the strain of BCG in the small Australian RCT evaluating the immunogenicity of three BCG strains.

These findings indicate that there is likely a direct relationship between the immunogenicity of the BCG strain administered, as determined by the objectively quantifiable immune responses, and the characteristics of the local skin reactions observed ten weeks after immunization. Aside from the substantial size differences, the number of infants that did not develop a BCG reaction is important. The authors did not report this, but when extracted from the Figure 3, it seems that 11% (6/54) of infants randomized to BCG-Denmark developed no reaction (non-reactors), while the number was 13% (7/54) for BCG-Japan and 19% (11/57) for BCG-Russia. Given the relatively small groups in the RCT, the differences are not significant, the Denmark versus Russia RR for no reaction being 0.58 (0.23-1.45), and the Japan versus Russia no-reaction RR being 0.67 (0.28-1.61). In an observational study from Uganda, effects of the BCG-Russia, BCG-Bulgaria and BCG-Denmark strains were evaluated at 12 months of age among infants vaccinated mainly during the first week of life. Both specific and non-specific cytokine responses, BCG scar frequency, adverse events, and mortality rates were presented.[20] Mimicking the results from the Australian RCT, infants immunized with BCG-Denmark showed the highest cytokine responses, most notably for IFN-γ, and a scar frequency of 93% versus 64% for BCG-Bulgaria and 52% for BCG-Russia.[20] Infants vaccinated with BCG-Denmark had more adverse events, indicating an association between the strain virulence and BCG skin reactions, adverse events and cytokine responses to both mycobacteria-specific and non-specific stimuli. Infants that had a BCG scar had higher IFN-γ and IL-13 responses to mycobacterial antigens, but no induction of non-specific cytokine responses when compared to those with no scar, indicating that induction of a scar might be associated with enhanced protection against TB. Futhermore, a recent study has evaluated specific and non-specific immune responses to BCG-Denmark, BCG-Russia, and BCG-Bulgaria in two cohorts from Nigeria and South Africa.[49] In the study, the infants were bled at 7, 15, and 36 weeks after birth, and blood samples from the cohorts were stimulated with BCG, Tetanus, and Pertussis antigens. The study concluded that BCG-Denmark mounted significantly higher frequencies of BCG-stimulated CD4+ T cell responses at all three points in time. The BCG strain also affected heterologous T cell responses to other vaccines and CD4+ T cells exposed to BCG-Bulgaria and BCG-Russia tended to accumulate in a naïve-like state with fewer polyfunctional cells. Contrary to this, BCG-Denmark was associated with a high proportion of polyfunctional CD4+ T cells that expressed IFN-γ, IL-2, and TNF-α. Finally, BCG-Denmark appeared to push the cells into a more differentiated memory state, and the results indicate that the BCG strain used has a profound effect on the magnitude and polyfunctionality of CD4+ T cell responses to unrelated heterologous vaccine antigens. Collectively, these novel data indicate that BCG-Denmark induces a more functional immune response in neonates and that BCG-Denmark is more immunogenic than the BCG-Bulgaria and BCG-Russia strains.[49]

A study from Brazil has compared the BCG-Denmark, BCG-Pasteur, and BCG-Moreau strains.[50] BCG-Moreau is produced and distributed exclusively in Brazil and is an early evolutionary strain, like BCG-Japan. BCG-Denmark, in contrast, is a late evolutionary strain that was separated from BCG-Pasteur at a later timepoint, and BCG-Denmark had thus accumulated additional genetic mutations after the distribution of the early strains. The Brazilian study featured blood samples from healthy BCG vaccinated adults and from unvaccinated neonates and focused on the in vitro immune responses in human mononuclear cells induced by infection with different BCG strains. The authors hypothesize that the ability to induce appropriate apoptosis represents a critical innate host response to

M. tuberculosis infection and that a strain which better induces such responses is more efficient against TB. The authors reported that BCG-Moreau was associated with significantly increased monocyte apoptosis in both healthy adults and newborn umbilical cord blood when compared to BCG-Pasteur and BCG-Denmark.[50] It was not detailed which BCG strain the healthy adults had received but it would likely be BCG-Moreau, which could affect their subsequent immune responses to specific BCG strains. Also, it was not detailed whether the parents of the neonates had been BCG vaccinated nor with which BCG strain, despite that maternal BCG scarring has been shown to affect infant immune responses.[51] The study concluded that the BCG-Moreau strain is more immunogenic than BCG-Pasteur and BCG-Denmark.

2.6 Efficacy of BCG strains against non-muscle-invasive bladder cancer

Following transurethral resection of the bladder, repeated intravesical instillations of BCG are routinely used as immunotherapy against non-muscle-invasive bladder cancer.[52] The mechanism of action is believed to be an attachment to or internalization of BCG by cancerous cells with secretion of cytokines and chemokines leading to the presentation of BCG and/or cancer cell antigens to immune system cells.[53] Different strains of BCG are used for this purpose, mainly based on the availability of the vaccines and tradition; only a few RCTs have investigated the efficacy of these different strains. Interestingly, whether maintenance BCG therapy (recommended) is provided or not seems to be a confounder of the effects by strain.[54] A trial testing BCG-Tice versus BCG-Connaught thus found BCG-Tice to be more effective than BCG-Connaught if maintenance therapy was given, while the Connaught/Tice RR for cancer recurrence was 0.68 (0.55-0.83) if maintenance therapy was not provided (Table 3).[54] Such differences illustrate that the BCG strain used might have important effects on treatment efficacy and that different strains might be superior in different treatment protocols.

Table 3. Comparison of BCG strains to treat non-invasive carcinoma of the bladder.

Maintenance BCG administered (recommended) - recurrence-free survival						Recurrence risk
RCT	Length of follow-up	Strain 1	Recurrence free/total (%)	Strain 2	Recurrence free/total (%)	Strain 1/Strain 2 RR (95% CI or P)
Witjes 2016[54]	5 years	Tice	136/205 (66%)	Connaught	319/560 (57%)	0.66 (0.47-0.93)
Mukherjee 1992[55]	5 years	Glaxo	5/12 (44%)	Pasteur	4/9 (42%)	P = 0.62
No maintenance BCG (*not* recommended) - recurrence-free survival						**Recurrence risk**
Witjes 2016[54]	5 years	Connaught	199/397 (50%)	Tice	381/937 (41%)	0.68 (0.55-0.83)
Rentsch 2014[56]	4 years	Connaught	53/71 (74%)	Tice	29/60 (48%)	0.64 (0.46-0.92)
Sengiku 2013[57]	2 year	Connaught	43/63 (69%)	Tokyo	48/66 (73%)	P = 0.90
Inamoto 2013[58]	1 year	Connaught	17/20 (84%)	Tokyo low dose	13/18 (72%)	P = 0.70
Fellows 1994[59]	3 months	Pasteur	18/46 (39%)	Evans	12/51 (24%)	0.60 (0.33-1.11)
Witjes 1996[60]	5 years	RIVM	72/134 (54%)	Tice	42/117 (36%)	0.67 (0.50-0.89)

Adapted from Professor Frank Shann (personal communication), data source D´Andrea et al.[52]

2.7 World Health Organization recommendations regarding BCG vaccination

The WHO recommends universal BCG vaccination to all neonates, including those with low birth weight in TB-endemic countries as part of the End TB Strategy, ideally within 24 hours after birth.[61] It is estimated that widespread use of BCG in routine infant vaccination programs could prevent 115,000 TB during the first 15 years of life deaths per birth cohort.[1] BCG given at birth is also believed to protect against *Mycobacterium lepra*, with experimental studies indicating a protective VE of 26% (14% to 37%).[62] WHO thus recently expanded the recommendation to include universal BCG vaccination in countries with a high burden of leprosy regardless of the TB-incidence.[61] Studies indicate that BCG might also prevent other nontuberculous mycobacteria including Buruli ulcer disease.[61]

There are several new TB vaccine candidates in the vaccine pipeline, and most are to be provided after BCG priming of newborns. It is therefore important to evaluate whether available BCG vaccine strain preparations vary in their priming and protective efficacy to ensure that a novel vaccine is not tested against or built upon a weak BCG strain.[63] The Strategic Advisory Group of Experts of the WHO thus recommends continued research into available BCG vaccine strains for better molecular characterization and quantification of product-specific aspects, as well as the conduct of comparative effectiveness studies to inform policymakers.[63]

BCG vaccination is contraindicated in HIV-infected persons unless anti-retroviral therapy has been started and the person is clinically and immunologically stable. BCG is also contraindicated in those with congenital cell-mediated or acquired immunodeficiency disease. For neonates born to women known to be HIV-infected but with unknown HIV status and no signs/symptoms suggestive of HIV infection, the neonate should be vaccinated, particularly if the mother is already receiving antiretroviral therapy.[1] This recommendation was already made in 1987.[64]

WHO has considered benefits of revaccination with BCG against TB and leprosy but does not support this practice due to limited evidence.[63] A large RCT from Malawi indicated that revaccination with BCG to BCG scar-positive infants gives further protection against leprosy, but no additional protection against TB.[65] WHO no longer regards the absence of a BCG scar after vaccination as indicative of a lack of protection and therefore does not recommend revaccination to scar-negative individuals.[63]

2.7.1 BCG dosage formulations and viable bacilli in different BCG strain preparations

The standard dose of reconstituted vaccine is 0.05 mL for infants aged <1 year and 0.1 mL for children >1 year and adults.[1] Using these doses, WHO estimates that approximately 10% of vaccine recipients do not develop a scar after vaccination. This is contradicted by trials conducted in Guinea-Bissau under well-controlled conditions: only 5% developed no scar across the three RCTs of BCG-Denmark versus no BCG[66], 2% in the more recent BCGIMED trial using BCG-Denmark and BCG-Japan[66] and 3% in the BCGSTRAIN I trial which included BCG-Denmark, BCG-Japan, and BCG-Russia.[67] These differences indicate that the experience of the vaccinator and the BCG strain provided plays a role for the subsequent BCG scar prevalence.

The current dosage recommendations are mainly based on a balance between ensuring an adequate immune response and limiting side-effects.[68] In WHO's BCG position paper from 2004, it is detailed that neonates have a higher risk of vaccine-induced suppurative lymphadenitis than older children, which is the reason why WHO recommends a reduced dose especially for neonates <30 days old.[69] The recommended dose of BCG to newborns was changed by WHO from 0.1 ml to 0.05 ml in 1993, which led to an increase in the percentage of children without BCG scars after vaccination in Chile.[70] This again prompted a small RCT evaluating tuberculin responses and BCG scar prevalence rates associated with different doses of BCG-Mérieux and BCG-Japan.[70] The study concluded that a neonatal dose of 0.1 ml BCG rather than 0.05 ml induces better scar and tuberculin responses. A study in India that randomized infants to 0.05 ml and 0.1 ml BCG-Denmark and conducted TST testing 10-12 weeks reached similar conclusions, e.g., that the lower dose was associated with fewer and smaller TST reactions and smaller BCG scars.[71]

Assessment of the immunogenetic potential of a BCG product can be performed by assessing CFUs in a standardized dose-preparation, estimating the number of viable bacilli in the BCG vaccine.[34] Historically, in vitro tests have been paired with clinical effects such as post-vaccination scar sizes, tuberculin conversions and surveillance of adverse events (regional lymphadenitis).[68]

The final BCG product is filled in containers according to a standard bacterial mass consisting of both living and dead bacilli. The viability (proportion of living versus dead bacilli) is an important characteristic of the final vaccine product. The extent of the local skin reaction at the injection site is thus proportional to the total bacterial mass due to local inflammatory processes, and the TST sensitivity is related to the number of culturable particles.[68] The number of culturable particles per dose is dependent also on the laboratory techniques employed. It can thus differ substantially even between laboratories producing the same strain of BCG.[68] Manufacturers might also be incentivized to reduce the number of viable bacilli, however, since the number of viable units likely influences the risk of local adverse events following a dose-response relationship.[72,73] The BCG-Japan strain comes with high viability and high resistance to freeze-drying; properties that have been prioritized by the Tokyo BCG laboratory, the producer of BCG-Japan, according to a historical review.[74]

A study evaluating immune responses to four different BCG-Tice doses tested very low (1.6×10^5 CFU), low (3.2×10^6 CFU), standard (1.6×10^8 CFU), or high-dose (3.2×10^8 CFU) BCG-Tice. A delayed-type hypersensitivity (TST) response occurred by eight weeks in 10% of persons given low or very low doses of BCG compared with 95% and 100% of persons given standard or high doses.[75] Similar results were reported in a calibration study from Senegal: a randomized, four-arm study provided tuberculin-negative children aged 8-10 years with either one of two new lots of the BCG-Mérieux vaccine, a standard dose of the WHO-reference strain (BCG-Japan) or BCG-Japan given at 1/10 of its normal concentration.[76] The percentage of subjects with a positive TST 10-12 weeks later was >96% for the three standard-dose (Mérieux or Japan) groups versus 82% in the low-dose BCG-Japan group ($p<0.001$). The mean induration diameters was also reduced for the low-dose group ($p<0.001$).[76] More children in the low-dose group also did not respond with a

BCG scar, and the mean scar diameters were smaller for those that received the low dose ($p<0.0001$) compared to the three other arms.[76]

If the immune response follows a dose-response pattern, as indicated by the studies described above, and TST responses and BCG scars are associated with enhanced survival, then the viable mycobacterial content in the resuspended vaccine strain preparation is likely very important. These data are available in leaflet package inserts and from the National Institute for Biological Standards and Control (NIBSC) for strains with established WHO reference agents (Table 4). Surprisingly, there are big differences in the content of viable mycobacteria among BCG strains, as measured by the number of cultural particles and the ATP content, the latter being proportional to the number of viable bacteria contained in the vaccine preparation.[77]

Table 4. Overview of cultural particles contained in BCG strains that are either established as WHO reference strains or with vaccine package insert information available.

Manufacturer	Mother strain (year)	Strain	Bacillary mass per ampoule	NIBSC: Cultural particles (SD) [ng ATP]	Package Insert: CFUs per 0.1 ml reconstituted vaccine
AJ Vaccines	BCG-Pasteur (1931)	Denmark	2 mg	7.3 million (0.9) [56][78]	2 to 8 million[79]
Green Signal Bio Pharma Limited	BCG-Denmark (2009)	Green Signal	NA	WHO Reference strain not established	2 to 8 million[80]
Intervax BB-NCIPD Ltd.	BCG-Russia (1950s)	Bulgaria	NA	WHO Reference strain not established	150,000 to 600,000[a,][81]
Japan BCG Laboratory	BCG-Pasteur-1173 (1925)	Japan	1 mg	49.4 million (5.9) [218][82]	30 million[b,][21]
Serum Institute of India Pvt. Ltd.	BCG-Russia (1924)	Russia	0.5 mg	3.4 million (0.5) [8][83]	200,000 to 800,000[84]
Fundação Ataulpho de Paiva	BCG-Pasteur (1925)	Moreau	10 mg	6.5 million (0.7) [25][85]	200,000[86]

Abbreviations: ATP, Adenosine Triphosphate; CFU, Colony-Forming Units; NIBSC, National Institute for Biological Standards and Control; NA, not available; ng; nanograms; SD, Standard Deviation.

[a]According to the package leaflet insert. According to[21], the CFU content is 4 million.

[b]According to Ritz et al.[21] p. 215, the CFU content per vial in BCG-Japan is 30 million while it is 2 to 8 million for BCG-Denmark. This is as indicated by the package leaflet, albeit per 0.1 ml reconstituted vaccine rather than per vial.

In the large MRC trial conducted in the UK, several batches of BCG-Denmark were used, and a count of the viable bacteria content of each batch was performed. For participants given a batch with <20 million viable units per mg, 76% had a positive TST response 3-5 months later versus 82% for 20-29 million viable units, 89% for 30-39 million viable units, and 97% for >40 million viable units.[18] A summary of studies evaluating the association between the BCG dose provided and BCG scars, scar size, and TST conversions is listed in Table 5.

Table 5. Studies that have evaluated the association between the BCG dose provided and the subsequent BCG scar formation, BCG scar sizes, or TST conversions.

Study	Design	Doses or BCG strains tested	Outcome(s)	Main conclusion(s)
Valenzuela 1998[70]	Double-blind RCT	BCG-Japan and BCG-Mérieux in doses of 0.05 and 0.1 ml	TST reactions and scar size	BCG-Japan > BCG-Mérieux, higher dose resulted in better scar and TST responses
Aggarwal 1995[71]	RCT	BCG-Denmark in doses of 0.05 ml or 0.1 ml at birth. A subgroup was given 0.1 ml at 4-6 weeks of age	TST positivity, TST reaction size, scar size	0.05 ml associated with significantly reduced TST positivity, reduced mean TST reaction size, and reduced mean scar size. No difference between 0.1 ml BCG at birth versus at 4-6 weeks
Lowry 1998[75]	RCT	Percutaneous placebo vs. very low, low, standard or high dose BCG-Tice to healthy adults with no history of BCG or TST reactivity	Erythema at the injection site after two weeks, TST reactivity and size by eight weeks, one year	100% of standard+high-dose vaccinees had erythema vs. 50% for low and 0% for very low dose and placebo. Standard + high-dose induced significantly more TSTs at eight weeks, one year. TST size correlated with dose. High-dose significantly associated w. lymphoproliferative responses, IFN-γ levels
Guérin 1999[76]	RCT	Two BCG-Mérieux lots, BCG-Japan, BCG-Japan diluted to 1/10 to TST-negative children aged 8-10 years	TST reactivity, mean TST size, BCG scar prevalence, and sizes	>96% positive TSTs for the three standard-dose (Mérieux or Japan) groups vs. 81.5% for low-dose ($p<0.001$). Mean induration diameter reduced ($p<0.001$) and fewer + smaller scars in the low-dose group.
MRC 1959[18]	Observational study within RCT	Different BCG-Denmark batches given to adolescents; viable bacterial count performed for each batch.	TST reactivity 3-5 months later	Low viability batch: 76% positive TSTs vs. 82% for medium viability batch, 89% for high and 97% for very high viability

While there seems to be substantial evidence to suggest that a dose-response relationship exists between the CFU content and beneficial immune responses, it is unlikely that the immunogenicity associated with different strains can be predicted solely from the CFU content and/or ATP residue in the vaccine vial preparation. A BCG vaccine with reduced CFU content may still show satisfactory properties regarding their ability to induce adequate sensitivity to TST, BCG scars, immune responses, and safety in humans.[87] Any packaging and processing differences (e.g., glutamate content, moisture content) and genetic differences between strains affecting the ability to survive and replicate in the host might play their separate roles. There is some evidence, however, that BCG seed lots that have been shown to have protective potency in laboratory animals and to induce TST conversion in humans are also effective in providing protection against TB in humans.[87] Quality tests of BCG strains in animals include protection tests, assessments of vaccination lesions, TST conversions, and assessment of the sensitizing efficacy measured as the average dose of vaccine that will convert a negative TST in guinea-pigs to a positive one.[87] This indicates that from a BCG strain and BCG batch quality perspective, the same factors could be used in humans to assess quality and efficacy.

As mentioned, it is of note that the content of viable mycobacteria in the dried BCG vaccine preparation and the TST conversion rate are positively correlated.[18,70,74–76] The strain differences detailed in Table 4 thus indicate that BCG-Denmark and BCG-Japan would likely be more immunogenic in terms of the rate of positive TST responses when compared to BCG-Bulgaria, BCG-Moreau, and BCG-Russia. Furthermore, the CFU and ATP content in BCG-

Japan surpasses BCG-Denmark and BCG-Green Signal approximately four-fold, and the implications of these vast differences for the protection against TB and the NSEs associated with different BCG strains merit further scrutiny.

One important study elucidating this question was recently published by Angelidou et al., who evaluated five licensed BCG formulations: BCG-Denmark, BCG-Japan, BCG-Russia, BCG-Bulgaria, and BCG-Tice.[88] The strains were tested in terms of bacterial viability, RNA content, mycobacterial membrane integrity, and innate immune activation properties (cytokine and chemokine production).[88] The study concluded that licensed BCG vaccines differ markedly in their content of viable mycobacteria and that BCG-induced cytokine production correlates with the CFU content in the BCG product. BCG-Russia and BCG-Bulgaria demonstrated substantially reduced bacterial growth and fewer colonies compared to BCG-Japan (>10-fold to >1000-fold lower growth depending on the growth medium). BCG-Russia also demonstrated significantly reduced membrane integrity and lower RNA content (indicating more dead cells), coupled with weaker whole blood IFN-γ induction, when compared to the other BCG strains.[88] The BCG-induced cytokine and chemokine production in whole human blood was CFU concentration-dependent, and BCG-Denmark and BCG-Japan induced higher levels of hematopoietic factors and Th1 cytokines when compared to BCG-Russia and BCG-Bulgaria.[88] The conclusion from the authors was that the viability (CFU) of licensed BCG strains correlated positively with the magnitude of proinflammatory cytokines and hematopoietic factors, suggesting that the absolute amount of viable organisms is the key trigger of the BCG-induced immune response.[88]

2.7.2 Route of BCG administration

When the first administration of BCG occurred on July 18, 1921, in Paris, France, it was provided as an oral dose to an infant born to a mother that had died of TB.[27] The use of subcutaneous and cutaneous routes was also tested at the time, but local reactions were objected to by caregivers to the children, and oral administration was thus continued. Today, most BCG vaccines are delivered via the intradermal route, as recommended by the WHO (Figure 4A+5B).[89] The main reason was that oral vaccination was found to be associated with reduced TST responses when compared to intradermal vaccination.[90]

An overview of other modes of administration is given in the Appendix (section 8.1).

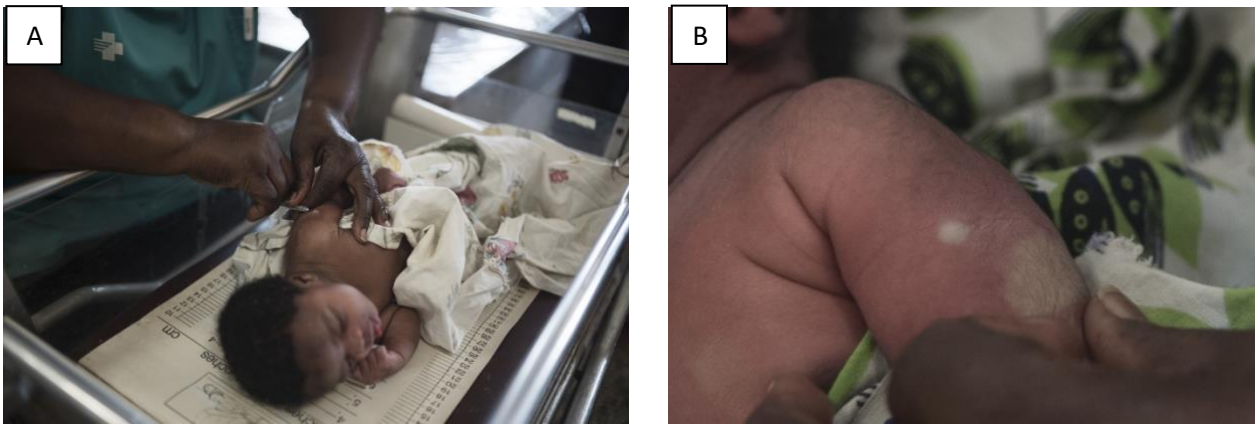


Figure 4. A: Intradermal BCG vaccination at HNSM in Bissau. B: The post-vaccination wheal immediately visible following the intradermal application of 0.05 ml BCG to a neonate. Photo credits: Sofia Busk.

2.8 Risk of adverse events after intradermal vaccination with BCG strains

While BCG vaccination is deemed safe for all neonates, including those born to HIV-positive mothers, it is associated with a risk of adverse events. Despite the vast number of vaccinated infants, reports of adverse events are relatively uncommon, however, and when serious reactions occur, they are often the result of vaccination of immuno-compromised individuals.[15] The BCG-Pasteur and BCG-Denmark strains are considered to cause more adverse events than other strains.[91] Following intradermal BCG vaccination, almost all infants develop a papule within 2-4 weeks at the injection site. The papule typically progresses to a small blister, which then ulcerates with purulent discharge. Repeated cycles of rupturing and partial healing then follow until the BCG ulcer dries up within 6-12 weeks to leave a permanent superficial scar.[92] This is the normal trajectory of BCG vaccination, but it can be mistaken for an adverse event if the mother/caretaker and medical professionals if sufficient information is not available.

The most common adverse event associated with intradermal BCG vaccination above the deltoid muscle is lymphadenitis in ipsilateral axillary lymph node(s) adjacent to the inoculation site. The risk of lymphadenitis is increased if the vaccine is injected too deeply and is thus coupled to the vaccination technique.[93] Lymphadenitis can either be simple (e.g., swelling of the lymph node) or, in rarer cases, suppurative with purulent discharge (Figure 5A+B).

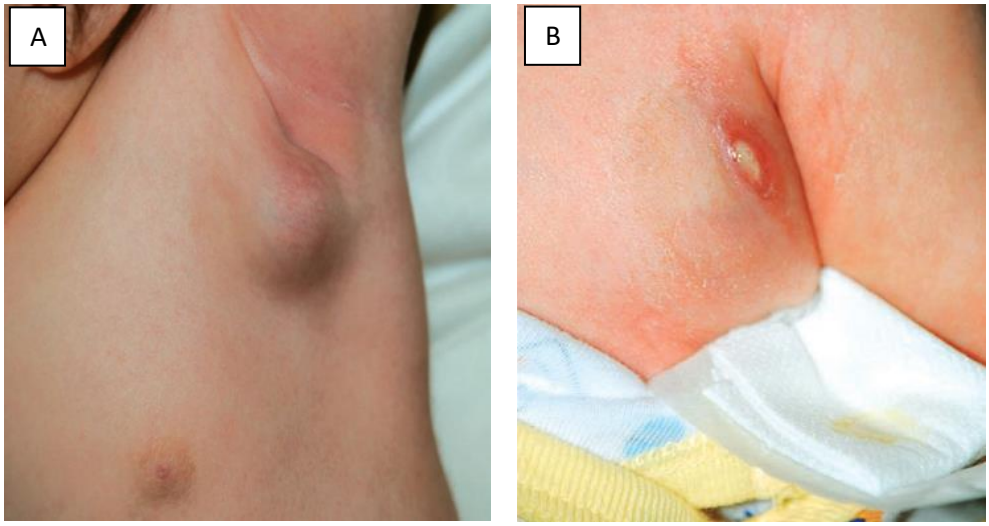


Figure 5. Simple and suppurative ipsilateral lymphadenitis following BCG vaccination.

Photo credit: Chan et al, Hong Kong Journal of Paediatrics.[94]

Some have considered simple regional lymphadenopathy without erythema or vesicle formation as a normal reaction to the vaccine.[95] The risk of suppurative lymphadenopathy is higher the younger the infant and the larger the dose given.[95] Such lesions can necessitate surgical excision, although a conservative approach and reassurance about the benign course of the condition are generally sufficient.[93,95]

The most severe adverse events associated with BCG vaccination are disseminated BCG disease and osteitis presenting 1 to 2 years after vaccination at birth. A Finnish study evaluating cases of osteitis among newborns that were BCG-vaccinated between 1960-1988

with different BCG strains reported an incidence of 6/100,000 using BCG-Glaxo, 7/100,000 using BCG-Gothenburg, and 37/100,000 using BCG-Denmark.[96] An increase in osteitis cases was also reported in Finland and Sweden after the production of BCG-Gothenburg was moved from Sweden to Statens Serum Institut in Copenhagen. This indicates that manufacturing procedures might influence the risk of adverse events associated with a BCG strain that has previously not been associated with adverse events.[97]

Numerous studies have reported an increase or decrease in the incidence of lymphadenitis after a change in the BCG strain provided under national vaccination programs; many following a change to the BCG-Denmark strain.[98–102] For example, a change from using primarily BCG-Bulgaria (genetically identical to BCG-Russia) to exclusively using BCG-Denmark in Georgia resulted in a 4-fold increase in BCG-associated lymphadenitis and an estimated incidence of 1.1/1,000.[102]

A study evaluating adverse events among 2,118 BCG-vaccinated children included in a Danish trial of BCG-Denmark registered no severe adverse reactions to BCG.[93] The study reported a 6.1/1,000 (3.3/1,000–10/1,000) incidence of regional lymphadenitis and a 4.7/1,000 (2.3/1,000–8.7/1,000) incidence of suppurative lymphadenitis (10 cases). The risk of suppurative lymphadenitis was nearly fivefold higher than had been indicated by the manufacturer. All cases of suppurative lymphadenitis were treated with conservative measures, and all infants recovered.[93]

Since BCG is a live vaccine, the circulation of mycobacteria in the body in the absence of an adequate immune response can lead to disseminated BCG disease (BCGitis), a disease that is mortal in 75%-86% of cases in HIV-infected infants.[91] While the risk of BCGitis for HIV-negative infants is under 5 per million vaccinated, it is substantially higher for HIV-positive infants.[103] In a study from South Africa, a country with a high HIV-disease burden, the risk of BCGitis among HIV-infected infants was evaluated. The study applied different vertical mother-to-child HIV transmission rate assumptions (between 5.4% to 10.4%), and the estimated incidence was between 778-1,013 per 100,000 HIV-infected, BCG-vaccinated infants, e.g., close to 1%.[103] A separate concern for HIV-infected children receiving BCG is immune reconstitution syndrome, which can occur in up to 15% of HIV-infected children that receive BCG.[91] Since universal HIV-screening before BCG vaccination is not feasible, the benefits of early BCG vaccination for protection against TB has been weighed against the risks and WHO maintains the recommendation that BCG is provided at birth to all HIV-negative infants and infants with unknown HIV-status.[1]

2.9 Early reports of specific and non-specific effects of BCG vaccination

After BCG had been distributed from France to several laboratories around the world and administered to more than 1 million infants in 1931-32, one of BCG's creators Albert Calmette (Figure 6), published a summary of the efforts and the preliminary results.[104] In the piece, Calmette attempted to counter skepticism, especially as to whether the mycobacterial attenuation could revert and become capable of attaining virulence and cause tuberculous lesions. At the time, TB was a widespread disease with high mortality, and BCG was mostly provided to infants from poorer households heavily exposed to TB; a mother or another close family member would often be infected. Calmette thus encouraged that BCG vaccination was performed at birth to prevent infants from being infected before vaccination. At the time, Calmette noted that not only did the mortality from TB drop markedly among those that were vaccinated, the all-cause mortality also dropped four-fold.[104] In a French cohort of 8,075 BCG-vaccinated and TB-exposed infants, the all-cause infant mortality was 4.6%, whereas overall infant mortality levels in France varied from 16%-25% at the time.[104] According to Calmette, similar patterns were reported from Romania, Sweden, Belgium, Holland, Spain, Greece, USA, Canada, and Uruguay.[104] The studies were not randomized but of observational design, and they are not referenced in his article. It has not been possible to find all the historical data for the present thesis.

Figure 6. Professor Albert Calmette (1861-1933) in 1930. Photo credit: Deschiens (Editor), Piccolati (Photographer).

Contemporary critics contested that the unvaccinated infants must have lived under less favorable conditions, e.g., the differences were caused by healthy-vaccinee bias:

"*Our own first statistics on this subject, published in 1926-1928, have been sharply criticized by some professional statisticians, principally by Greenwood (London), Rosenfeld and Gotzl (Vienna). They reproached us with not taking into consideration the "tables of life" and methodological rules, which is justifiable criticism. But they unjustly reproached us with choosing infants to be vaccinated among those belonging to better-controlled families who were protected by better precautions against infection, whereas the non-vaccinated lived under less favourable conditions. This criticism is unjust and erroneous, for we took the utmost care to deal with exactly comparable groups of infants.*"[104]

Based on the reports from a series of countries indicating broad health benefits associated with BCG vaccination, Calmette speculated the following:

"*How can this difference between general mortality in vaccinated and in non-vaccinated groups be explained ? (...) does the harbouring of BCG; followed by its digestion and elimination, confer on the organism a special aptitude to resist other infections which are so frequent in young children?*"[104]

A summary of Calmette's early data from the provision of BCG to the first million infants, of which about 2/3 were from France or French colonies, was published in 1932 in the Journal of the American Medical Association.[105] Among 443,656 infants from 46 countries outside of France, the infant death rate was 7.9% among BCG-vaccinated children and 15.3% among non-vaccinated infants.[105] As Calmette died in 1933 he did not live to see his vaccine receive the recognition it deserved.

Swedish data from the introduction of BCG in the northernmost Swedish province in 1927 was published in French by Carl Näslund in 1932.[106] BCG was provided at birth mainly to infants that were judged to be at high risk of TB and thus often those from poorer households. The cohort included 20,012 infants, of which 5,659 received BCG (5,656 of which were vaccinated within five days after birth). The overall mortality in the cohort was 8.7% (1,726/20,012) between 1927 and 1931. Since it was not feasible to conduct autopsies on all infants that died, Näslund both evaluated the causes of death based on medical records and the all-cause mortality for vaccinated and unvaccinated infants.

Since it was logistically difficult to provide BCG within the first five days, Näslund reported mortality numbers between 0-5 days after birth and from 5 days and up to 4 years of age. The mortality in the first five days was 0.1% (3/5,656) among vaccinated neonates versus 2.4% (339/14,353) for unvaccinated infants.[106]

In the neonatal period (0 to 28 days), the all-cause neonatal mortality was 0.6% (33/5,659) among BCG-vaccinated infants and 4.5% (650/14,353) among the unvaccinated (Table 6).[106]

Table 6. Causes of death by BCG vaccination status and period of follow-up among 5,659 BCG-vaccinated and 14,353 BCG-unvaccinated infants, Norrbotten, Sweden, 1927-31.

	Neonatal deaths n (%)		Infant deaths after the neonatal period n (%)		Deaths between 1 to 4 years of age n (%)	
Cause of death	BCG (n=5,659)	No BCG (n=14,353)	BCG (n=5,626)	No BCG (n=13,703)	BCG (n=5,488)	No BCG (12,979)
Tuberculosis	0 (0.0%)	5 (0.0%)	2 (0.0%)	23 (0.2%)	3 (0.1%)	36 (0.3%)
Meningitis	0 (0.0%)	2 (0.0%)	5 (0.1%)	10 (0.1%)	8 (0.1%)	4 (0.0%)
Pneumonia	3 (0.1%)	16 (0.1%)	24 (0.4%)	105 (0.8%)	8 (0.1%)	32 (0.2%)
Bronchitis	2 (0.0%)	16 (0.1%)	16 (0.3%)	64 (0.5%)	5 (0.1%)	12 (0.1%)
Influenza	2 (0.0%)	4 (0.0%)	8 (0.1%)	39 (0.3%)	2 (0.0%)	11 (0.1%)
Pertussis	0 (0.0%)	1 (0.0%)	10 (0.2%)	48 (0.4%)	1 (0.0%)	8 (0.1%)
Diphtheria, measles, scarlet fever, erysipelas or sepsis	4 (0.1%)	13 (0.1%)	4 (0.1%)	19 (0.1%)	1 (0.0%)	10 (0.1%)
Digestive tract infection	2 (0.0%)	21 (0.1%)	8 (0.1%)	59 (0.4%)	0 (0.0%)	4 (0.0%)
Hyperventilation	0 (0.0%)	14 (0.1%)	10 (0.2%)	39 (0.3%)	1 (0.0%)	7 (0.1%)
Congenital conditions	14 (0.2%)	360 (2.5%)	6 (0.1%)	98 (0.7%)	2 (0.0%)	4 (0.0%)
Other non-infectious diseases	2 (0.0%)	61 (0.4%)	12 (0.2%)	58 (0.4%)	4 (0.1%)	13 (0.1%)
Cause unknown	4 (0.1%)	137 (1.0%)	33 (0.6%)	162 (1.2%)	5 (0.1%)	20 (0.2%)
Total	33 (0.6%)	650 (4.5%)	138 (2.5%)	724 (5.3%)	40 (0.7%)	161 (1.2%)

In the infant period (and after the neonatal period), mortality was 2.5% (138/5,626) for BCG-vaccinated versus 5.3% (724/13,703) for unvaccinated. Between 1 to 4 years, the all-cause mortality was 0.7% (40/5,488) among BCG-vaccinated infants and 1.2% (161/12,979) among unvaccinated (Figure 7).[106]

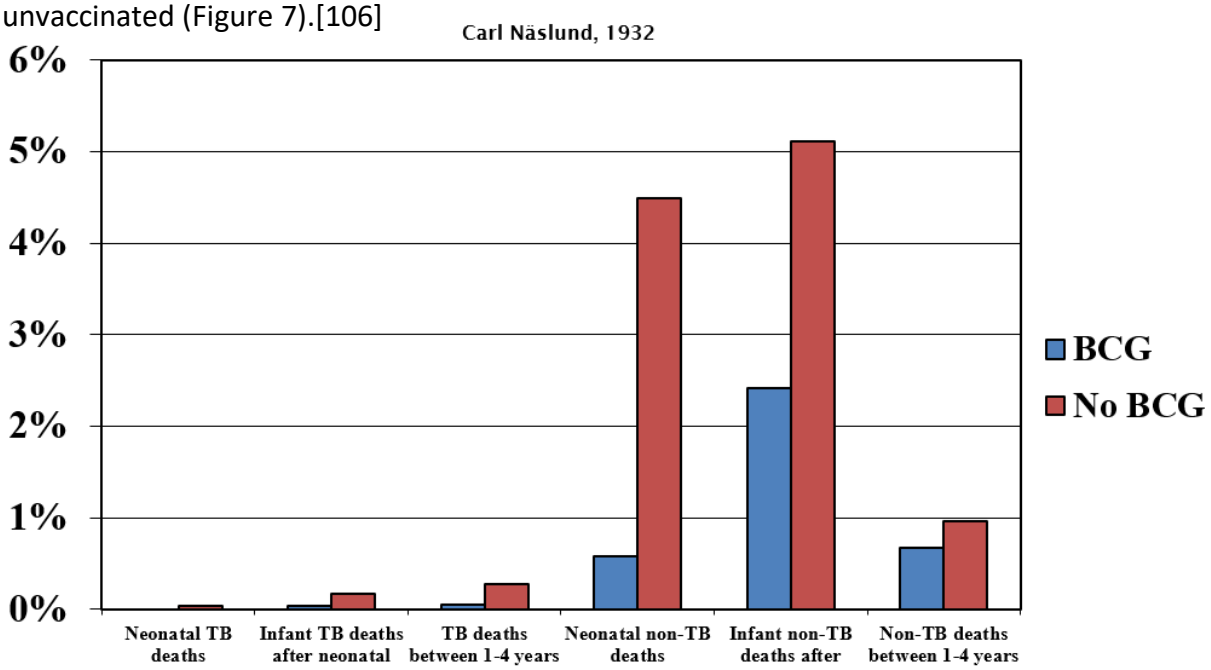


Figure 7. Distribution of TB deaths and non-TB deaths by BCG vaccination status and period of follow-up, 1927-1931, Norrbotten, Sweden.

Not all deaths could be ascribed a cause; 20% (42/211) of the total deaths between 0-4 years in the vaccinated group and 21% (319/1,535) in the unvaccinated group were of unknown cause. Furthermore, 10% (22/211) of the total 0-4-year deaths among vaccinated versus 30% (462/1,535) of the deaths among unvaccinated were determined to be due to congenital conditions, a high number for both groups. This discrepancy suggests that a healthy-vaccinee bias might have influenced the vaccinators, e.g., that neonates deemed too frail were not vaccinated. It also seems likely that infants could have died before they could be vaccinated (survival bias). Both sources of bias would favor the vaccine in the comparison. Since 2.4% (339/14,353) of the unvaccinated infants had died already within the first five days and that it was challenging to provide BCG immediately after birth, survival bias likely influenced the data, especially for the first days after birth. E.g., some newborns died before they had a chance to be BCG vaccinated and therefore remained in the unvaccinated group. Finally, 8.5% (18/211) of deaths among the vaccinated and 9.3% (142/1,535) of deaths among the unvaccinated were deemed to be caused by non-infectious diseases.[106] Interpreting the mortality differences between vaccinated and unvaccinated infants, Näslund speculated (French):

"*On pourrait évidemment être tenté de trouver une explication de cette mortalité plus basse des enfants vaccinés dans l'idée que le vaccin BCG provoque* ***une immunité non spécifique****. S'il en était ainsi, les décès causés par les différentes maladies infectieuses devraient être relativement plus nombreux parmi les enfants non vaccinés que parmi les enfants vaccinés. Tel ne fut cependant pas le cas et le tableau IV fait parfaitement ressortir ceci."*[106]

English translation:

"*One might of course be tempted to find an explanation for this lower mortality of vaccinated children in the belief that the BCG vaccine causes* ***non-specific immunity****. If this were the case, the deaths caused by the different infectious diseases should be relatively more numerous among the unvaccinated children than among the vaccinated children. This was not the case, however, and Table IV makes it perfectly clear.*"

Näslund then examined the mortality data by cause of death and noted that a substantial share of the unvaccinated infants died from congenital causes. Unfortunately, he did not conduct a separate analysis of the data strictly focusing on infectious disease deaths. I recently did the analysis.

By excluding all deaths deemed by Näslund to be caused by either congenital conditions, non-infectious diseases and unknown causes, conservative estimates can be obtained. These might underestimate the true effect of BCG on infectious diseases due to the uncertainty related to the diagnostic accuracy in this large cohort. Using this approach and assuming that deaths due to Spasmophilia (hyperventilation) were caused by infection, the neonatal mortality from infectious diseases was 0.2% (13/5,639) among BCG-vaccinated infants and 0.7% (92/14,063) among unvaccinated (Table 7, deaths due to other causes censored). The corresponding neonatal BCG versus no BCG RR was thus 0.35 (0.20-0.63) (Fisher's 2-sided exact test).

Table 7. Causes of death by BCG vaccination status and period of follow-up among 5,659 BCG-vaccinated and 14,353 BCG-unvaccinated infants, Norrbotten, Sweden, 1927-31.

	Neonatal deaths n (%)		Infant deaths after the neonatal period n (%)		Deaths between 1 to 4 years of age n (%)	
Cause of death	BCG (n=5,659)	No BCG (n=14,353)	BCG (n=5,626)	No BCG (n=13,703)	BCG (n=5,488)	No BCG (12,979)
Infectious conditions	13 (0.2%)	92 (0.7%)	87 (1.6%)	406 (3.0%)	29 (0.5%)	124 (1.0%)
BCG/unvaccinated RR	0.35 (0.20-0.63)		0.51 (0.41-0.65)		0.55 (0.37-0.83)	
Congenital conditions	14 (0.2%)	360 (2.6%)	6 (0.1%)	98 (0.7%)	2 (0.0%)	4 (0.0%)
BCG/unvaccinated RR	0.10 (0.06-0.17)		0.15 (0.06-0.33)		1.18 (0.22-6.42)	
Non-infectious diseases	2 (0.0%)	61 (0.4%)	12 (0.2%)	58 (0.4%)	4 (0.1%)	13 (0.1%)
BCG/unvaccinated RR	0.08 (0.02-0.33)		0.49 (0.26-0.91)		0.72 (0.24-2.22)	
Unknown	4 (0.1%)	137 (1.0%)	33 (0.6%)	162 (1.2%)	5 (0.1%)	20 (0.2%)
BCG/unvaccinated RR	0.07 (0.03-0.20)		0.48 (0.33-0.70)		0.59 (0.22-1.57)	
All causes	33 (0.6%)	650 (4.5%)	138 (2.5%)	724 (5.3%)	40 (0.7%)	161 (1.2%)
BCG/unvaccinated RR	0.13 (0.09-0.18)		0.46 (0.39-0.56)		0.59 (0.42-0.83)	

Note: Deaths from other causes are censored in each subgroup.

Similarly, for infant infectious disease deaths after the neonatal period, the mortality was 1.6% (87/5,575) for BCG-vaccinated infants versus 3.0% (406/13,385) for unvaccinated infants, the BCG/no BCG RR being 0.51 (0.41-0.65) (Table 7). Between 1-4 years, the mortality risk was 0.5% (29/5,477) versus 1.0% (124/12,942) and the BCG/no BCG RR=0.55 (0.37-0.83) (Figure 8). It is quite clear from Table 7 that there was a substantial

overrepresentation of deaths due to congenital conditions, non-infectious diseases, and unknown causes in the unvaccinated group during infancy. But the finding of a substantial beneficial effect of BCG in all three age groups including from 1-4 years of age, when the other causes no longer had a substantial impact on mortality, indicates that BCG was likely associated with long-term beneficial effects on deaths from infectious diseases in this cohort. This effect cannot be explained entirely by healthy-vaccinee bias and survival bias since the importance of these effects should decline over time. Yet, the apparent beneficial effect of BCG remained stable and similar through the infant period (excluding the neonatal period) and the subsequent three years of life.

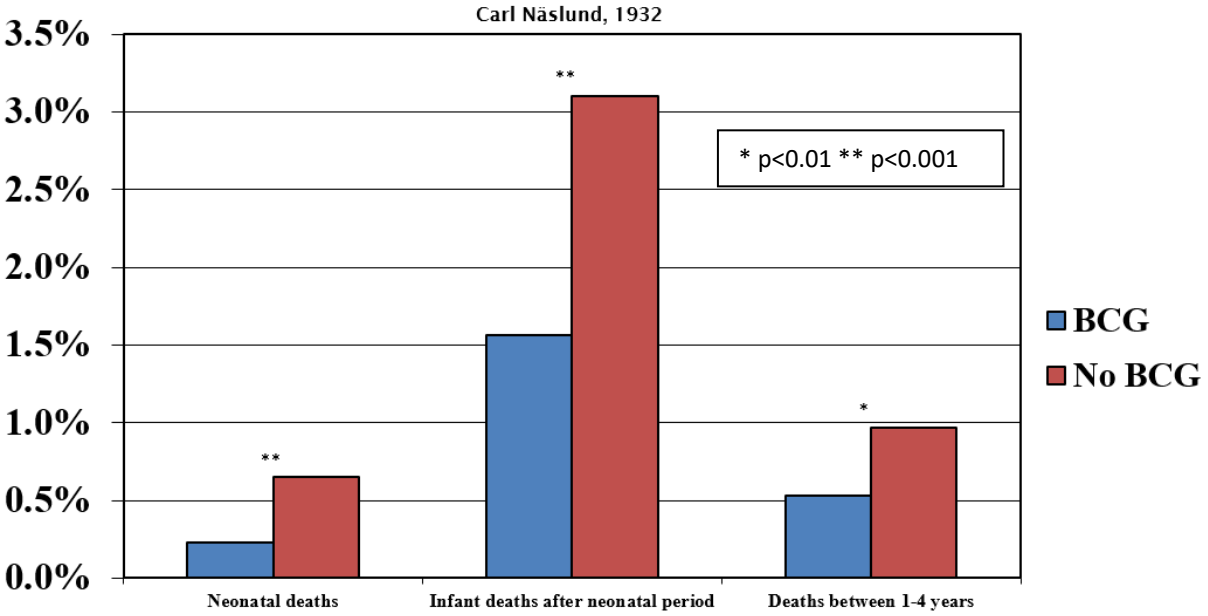


Figure 8. Distribution of deaths from infectious diseases by BCG vaccination status and period of follow-up, 1927-1931, Norrbotten, Sweden.

Unfortunately, these initial data indicating BCG-induced beneficial NSEs on the severity of infectious diseases were not pursued until many decades later. Aside from these early observational data, six controlled trials with different designs that investigated the effects of BCG and included data on overall mortality were published between 1948 and 1961. After the first studies from Guinea-Bissau indicating a beneficial effect of BCG had appeared, a meta-analysis published in 2010 of the six historical trials indicated that BCG was associated with a 25% (6% to 41%) reduction in mortality when compared to no BCG.[107]

2.10 Discovering NSEs and the background for investigating BCG's NSEs in Bissau

The paradigm-changing concept of *non-specific effects* of vaccines was first proposed Professor Peter Aaby, who had come to Guinea-Bissau in 1978 and witnessed a severe measles epidemic with a case-fatality rate of 25% among children under three years of age.[107] Aaby had conducted a census and established that the under-five mortality in Bissau was almost 500/1,000. Importantly, the very high mortality was not caused by malnutrition. For example, there was no difference in the nutritional status of infants that had died from measles compared to other children in the community, which contradicted

the existing medical paradigm of the time.[108] Instead, the main determinants for measles mortality was the intensity of exposure.[109] Index cases (that had contracted measles in the community) thus had lower mortality than those infected at home, where the intensity of exposure was higher.[110] Another important observation was that measles infection was more severe when passed on by the opposite sex.[111] This was discovered serendipitously due to the translation by none other than my father of “sibling” to “irmão” in Portuguese (which has no word for a *sibling*; irmão means brother). The translation made the investigators go back to the original records to check the sex of the infants and make the discovery.

After having arranged for the provision of measles vaccines to children that had not already had measles infection and that were present for an anthropometric survey, Aaby noted that vaccination was associated with a substantial reduction in mortality despite that there was little circulating measles after the epidemic.[112] A recent reanalysis estimated the mortality reduction to have been 70% (27% to 88%).[113] This led to two possibilities: either measles infection was associated with long-term immune suppression and thus higher mortality relative to those that had not been infected (and therefore were vaccinated), or measles vaccination was associated with beneficial NSEs. To answer this question, ten cohort studies with data on mortality and measles infections among unvaccinated infants has been analyzed. When excluding all cases of measles (and thus the possible short-term and long-term consequences on the immune system) in the analysis, the protective effect on mortality associated with measles vaccination was unaffected.[114]

In an RCT conducted in Guinea-Bissau, it was tested whether the provision of an additional dose of MV provided at 4.5 months (two-dose group) versus MV at nine months (provided to all infants) was associated with a 25% reduction in mortality between 4.5-36 months. Between 4.5 months to 3 years of age, the MRR for infants that received two doses of MV (at 4.5 and 9 months) versus MV at nine months (standard practice) was 0.78 (0.59-1.05); for girls, the same MRR was 0.64 (0.42-0.98) versus 0.95 (0.64-1.42) for boys.[115] There was a measles epidemic at the beginning of the trial, but the differential pattern in mortality for infants that received two doses of MV versus one dose was the same in the cohort included after the measles epidemic and the cohort included during the epidemic.[115] In the per-protocol analysis, two doses of MV was associated with a 30% (6% to 48%) lower mortality between 4.5-36 months, and the reduction was 26% (0% to 45%) when measles cases were censored in the analysis. Hence, the mortality reduction was mostly non-specific, and the theory of immune suppression after measles infection again fails to explain the findings.[115] The theory has nevertheless recently reemerged.[116]

An analysis of hospitalization patterns between 4.5 to 9 months also indicated that receiving early MV was associated with a reduced risk of hospital admission, the MV versus no-MV RR being 0.70 (0.52-0.95); the RR was 0.53 (0.32-0.86) for girls and 0.86 (0.58-1.26) for boys.[117] These findings have important implications since measles vaccination may be stopped or the number of doses could be reduced, if the disease is eradicated.

The discovery of non-specific effects related to measles vaccination and the establishment of the BHP HDSS paved the way for the study of the long-forgotten NSEs of BCG.

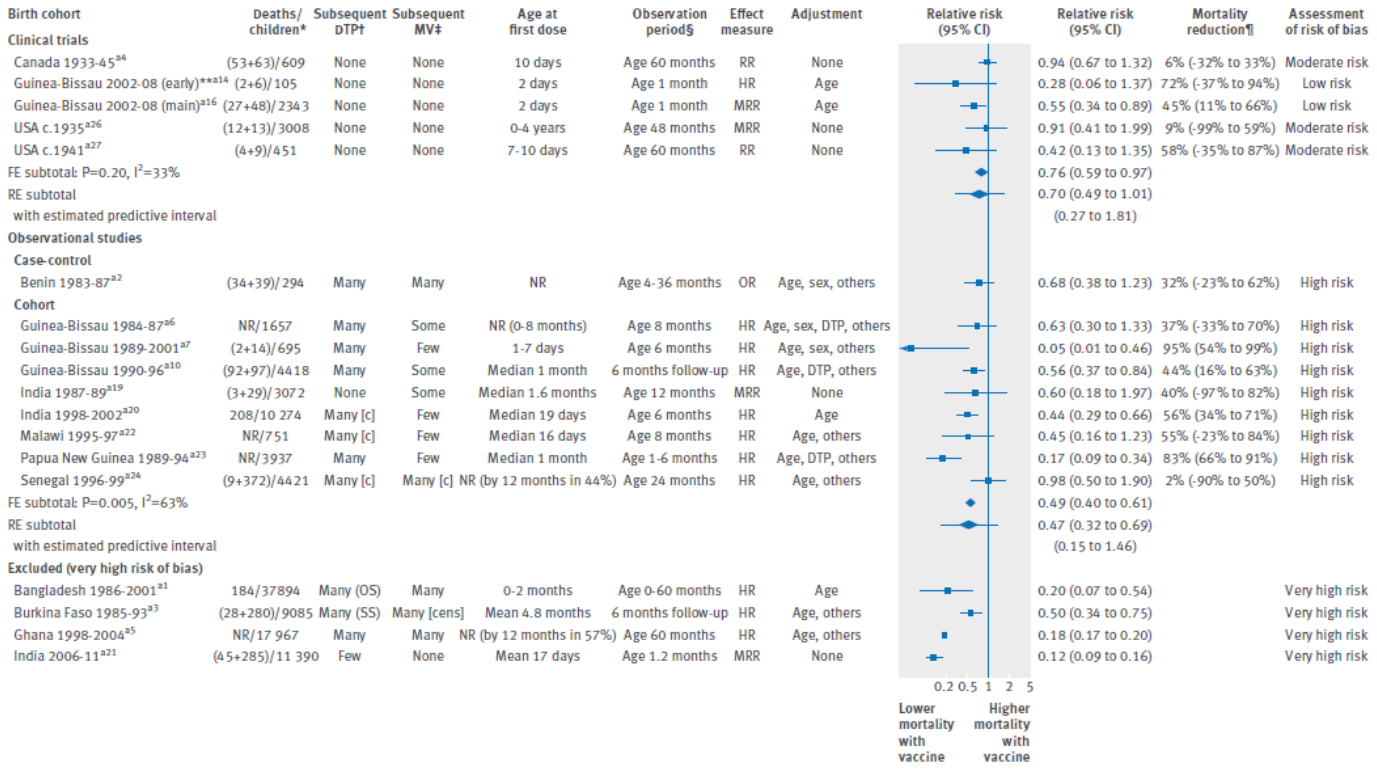
2.10.1 Observational studies evaluating effects of BCG vaccination, BCG scars, and TST responses

When attempting to evaluate the effects of a vaccine on subsequent health outcomes using observational data, it is important to bear in mind that vaccinators generally refrain from vaccinating overtly sick or underweight infants. Parents might also be hesitant to have their child vaccinated in such a situation. As mentioned, this phenomenon is referred to as the *healthy-vaccinee bias*.[118] Relevant anthropometric data points such as weight, MUAC, and socio-economic background factors should be taken into consideration to ensure that the group of vaccinated infants is comparable to the unvaccinated, or analyses should be conducted with adjustment for known differences. Also, survival bias is an important issue, since data on vaccinations is never complete in longitudinal study sites.[119] The vaccine information might be updated on a certain day for an infant, who, one month later, might receive a vaccine and subsequently die. If the vaccination card was not inspected again shortly before the death or post-mortem, such an infant might end up analyzed in the non-vaccinated group (misclassification bias), even though the infant had been vaccinated. Applying survival analysis with interval-fixed vaccination status (landmark approach) can alleviate this problem while providing a conservative estimate of the true effect of the vaccine on mortality.[119]

In Bissau, the observations regarding measles meant that BHP started investigating whether other vaccines also have non-specific effects. A pivotal observational study was published in the year 2000 and included data on 10,298 children born between 1990-96 in the rural areas of Guinea-Bissau, where mortality was high.[120] The study evaluated the effects of MV, BCG, DTP, and polio vaccination (practically always given together with DTP). While receiving any of the vaccines compared to not receiving a vaccine was associated with an MR of 0.74 (0.53-1.03), there was a remarkable discrepancy between the effects of the different vaccines. Receiving MV versus no MV was thus associated with an MR of 0.48 (0.27-0.87), and receiving BCG versus no BCG was associated with an MR of 0.55 (0.36-0.85), yet receiving DTP/polio was associated with an MR of 1.84 (1.10-3.10).[120] The very opposite tendencies between the different vaccines make selection bias unlikely since such a bias should have affected the different vaccines in a similar way. Controlling for differences in cultural and social background factors also did not affect the mortality estimates for the different vaccines.

In a recent large observational study involving 39,421 children, of which 84% had received neonatal BCG, neonatal BCG was beneficial for both TB-exposed and TB-unexposed infants, the BCG/no BCG HR up to three years of age being 0.54 (0.45-0.65).[121]

A series of observational studies from low-income countries summarized by Higgins et al.[122] have shown a similar pattern, e.g., that BCG vaccination is associated with reduced all-cause mortality. The fixed effects meta-analysis estimate across eight cohort studies and one case-control study was 0.47 (0.32-0.69) (Figure 9).



Birth cohort	Deaths/ children*	Subsequent DTP†	Subsequent MV‡	Age at first dose	Observation period§	Effect measure	Adjustment	Relative risk (95% CI)	Relative risk (95% CI)	Mortality reduction¶	Assessment of risk of bias
Clinical trials											
Canada 1933-45[a24]	(53+63)/609	None	None	10 days	Age 60 months	RR	None		0.94 (0.67 to 1.32)	6% (-32% to 33%)	Moderate risk
Guinea-Bissau 2002-08 (early)**[a14]	(2+6)/105	None	None	2 days	Age 1 month	HR	Age		0.28 (0.06 to 1.37)	72% (-37% to 94%)	Low risk
Guinea-Bissau 2002-08 (main)[a16]	(27+48)/2343	None	None	2 days	Age 1 month	MRR	Age		0.55 (0.34 to 0.89)	45% (11% to 66%)	Low risk
USA c.1935[a26]	(12+13)/3008	None	None	0-4 years	Age 48 months	MRR	None		0.91 (0.41 to 1.99)	9% (-99% to 59%)	Moderate risk
USA c.1941[a27]	(4+9)/451	None	None	7-10 days	Age 60 months	RR	None		0.42 (0.13 to 1.35)	58% (-35% to 87%)	Moderate risk
FE subtotal: P=0.20, I^2=33%									0.76 (0.59 to 0.97)		
RE subtotal									0.70 (0.49 to 1.01)		
with estimated predictive interval									(0.27 to 1.81)		
Observational studies											
Case-control											
Benin 1983-87[a2]	(34+39)/294	Many	Many	NR	Age 4-36 months	OR	Age, sex, others		0.68 (0.38 to 1.23)	32% (-23% to 62%)	High risk
Cohort											
Guinea-Bissau 1984-87[a6]	NR/1657	Many	Some	NR (0-8 months)	Age 8 months	HR	Age, sex, DTP, others		0.63 (0.30 to 1.33)	37% (-33% to 70%)	High risk
Guinea-Bissau 1989-2001[a7]	(2+14)/695	Many	Few	1-7 days	Age 6 months	HR	Age, sex, others		0.05 (0.01 to 0.46)	95% (54% to 99%)	High risk
Guinea-Bissau 1990-96[a10]	(92+97)/4418	Many	Some	Median 1 month	6 months follow-up	HR	Age, DTP, others		0.56 (0.37 to 0.84)	44% (16% to 63%)	High risk
India 1987-89[a19]	(3+29)/3072	None	Some	Median 1.6 months	Age 12 months	MRR	None		0.60 (0.18 to 1.97)	40% (-97% to 82%)	High risk
India 1998-2002[a20]	208/10 274	Many [c]	Few	Median 19 days	Age 6 months	HR	Age		0.44 (0.29 to 0.66)	56% (34% to 71%)	High risk
Malawi 1995-97[a22]	NR/751	Many [c]	Few	Median 16 days	Age 8 months	HR	Age, others		0.45 (0.16 to 1.23)	55% (-23% to 84%)	High risk
Papua New Guinea 1989-94[a23]	NR/3937	Many	Few	Median 1 month	Age 1-6 months	HR	Age, DTP, others		0.17 (0.09 to 0.34)	83% (66% to 91%)	High risk
Senegal 1996-99[a24]	(9+372)/4421	Many [c]	Many [c]	NR (by 12 months in 44%)	Age 24 months	HR	Age, others		0.98 (0.50 to 1.90)	2% (-90% to 50%)	High risk
FE subtotal: P=0.005, I^2=63%									0.49 (0.40 to 0.61)		
RE subtotal									0.47 (0.32 to 0.69)		
with estimated predictive interval									(0.15 to 1.46)		
Excluded (very high risk of bias)											
Bangladesh 1986-2001[a1]	184/37894	Many (OS)	Many	0-2 months	Age 0-60 months	HR	Age		0.20 (0.07 to 0.54)		Very high risk
Burkina Faso 1985-93[a3]	(28+280)/9085	Many (SS)	Many [cens]	Mean 4.8 months	6 months follow-up	HR	Age, others		0.50 (0.34 to 0.75)		Very high risk
Ghana 1998-2004[a5]	NR/17 967	Many	Many	NR (by 12 months in 57%)	Age 60 months	HR	Age, others		0.18 (0.17 to 0.20)		Very high risk
India 2006-11[a21]	(45+285)/11 390	Few	None	Mean 17 days	Age 1.2 months	MRR	None		0.12 (0.09 to 0.16)		Very high risk

Forest plot for BCG and all cause mortality. c=event was censored in analysis; FE=fixed effect meta-analysis method; HR=hazard ratio; MRR=mortality rate ratio; OR=odds ratio; OS=often received simultaneously with DTP; RE=random effects meta-analysis method; RR=relative risk; SS=sometimes received simultaneously with DTP. *(BCG deaths+non-BCG deaths)/total children or total deaths/total children. †Proportion of children likely to receive DTP during period of observation. ‡Proportion of children likely to receive MCV during period of observation. §Period of observation applicable to result presented in forest plot, aiming to capture effect of BCG with minimal impact of subsequent vaccinations; full study may have had longer follow-up. ¶Computed as (1–RR)×100%; non-negative number describes proportion of deaths prevented by vaccine; negative number reflects higher death rate among vaccinated children (for example, if vaccine efficacy is −100%, then an additional 100% of deaths that would have occurred without vaccine would occur with vaccine). **Early phase of trial stopped prematurely because of faulty randomisation procedure in one centre. ††(Subsequent) main trial phase with larger sample size (both phases in low birthweight infants only). In two cohort studies with "none" as adjustment for confounding, unadjusted rate ratios were computed from rates presented in article.

Figure 9. Clinical trials and observational studies included in the review of NSEs conducted at the request of WHO's SAGE.

The above BCG data was reported in the statistical analysis commissioned by WHO's Strategic Advisory Group of Experts on Vaccination (SAGE) to evaluate whether the evidence concerning non-specific effects of vaccines is sufficient to warrant adjustments in the routine immunization schedule and/or further research.[122] The authors estimated that MV is associated with a halving in all-cause mortality risk, RR=0.51 (0.42-0.63) and that BCG was likewise associated with an RR of 0.52 (0.33-0.82) in the more recent LBW RCTs. BCG was associated with an RR of 0.47 (0.32-0.69) in the nine observational studies included, while DTP vaccination was associated with an RR of 1.38 (0.92-2.08).[122] The unlikely pattern of opposite effects between MV and BCG (beneficial) and DTP (detrimental) reported in the landmark study from Bissau was thus also evident in WHO's meta-analysis. The authors concluded that the data does not support immediate changes in either the choice of vaccines, timing nor the sequence of immunizations provided routinely to infants and children. But it was strongly recommended to conduct further studies into the NSEs of vaccines.[122] Several RCTs evaluating NSEs of BCG or BCG strains in Bissau[123–125], Uganda[126,127], and India[128] are currently recruiting participants or awaiting publication. We do not know of RCTs that are either planned or enrolling to test the NSEs of DTP, despite the urgency of such a trial.

Aside from evaluating the effects of BCG in comparisons of vaccinated versus unvaccinated individuals, there is also the possibility to evaluate the effects among infants that develop the characteristic BCG scar on the skin (Figure 10-Figure 11) versus those that do not.

Figure 10. Assessment of a BCG scar at six months of age at an HDDS home visit in the BCGSTRAIN I study in Bissau. Photo credit: Sofia Busk.

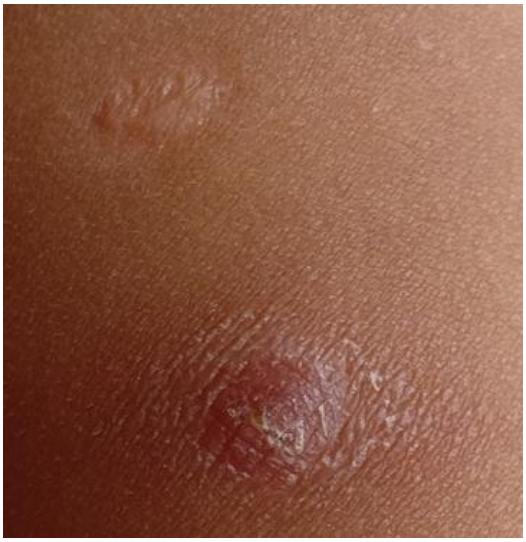


Figure 11. A BCG scar at one year of age (top left corner) from neonatal vaccination and a BCG reaction (bottom center) approx. 2 months after booster vaccination with BCG. Photo: The author.

The main determinants for the formation of a BCG scar are the vaccination technique and the strain of BCG provided.[66,67,129–131] Additionally, successful immunization with BCG often leads to a positive immune response after the application of a standardized TST (Figure 12).

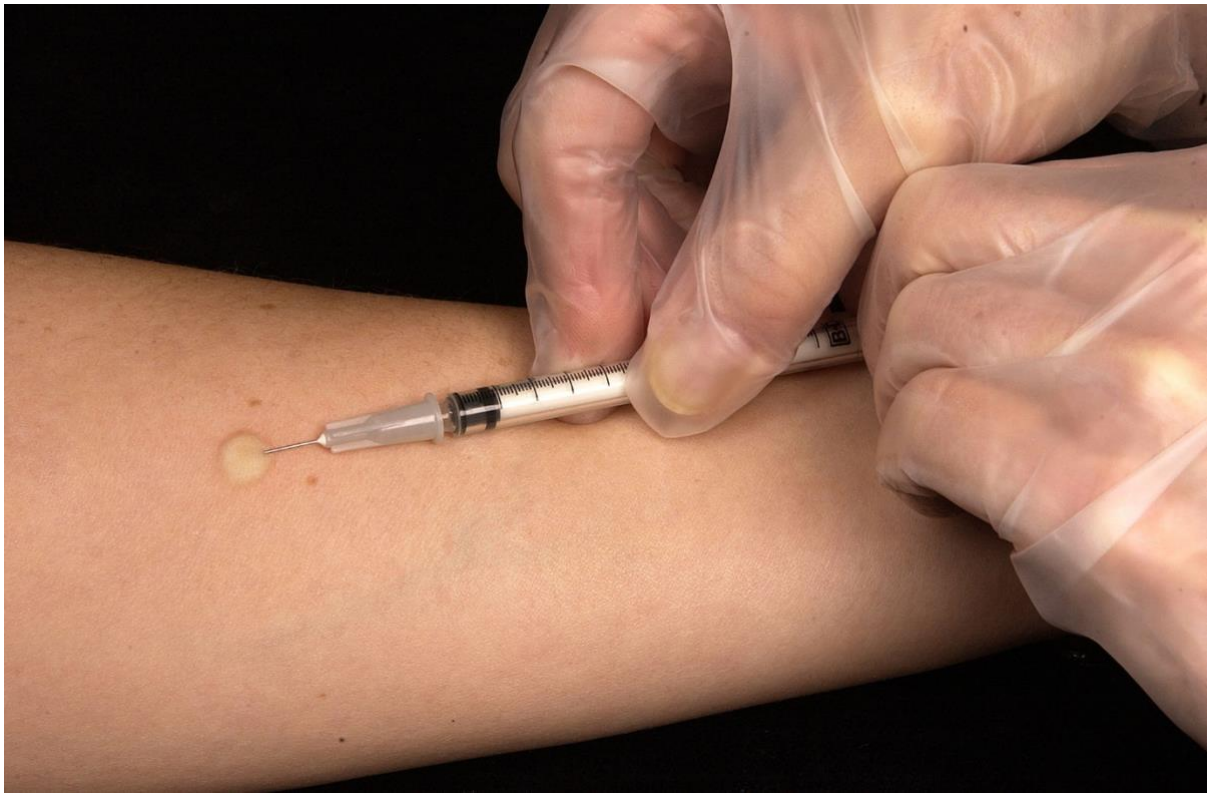


Figure 12. Application of a TST in an adult's forearm: 0.1 ml is injected intradermally to form a wheal of 6 to 10 mm. Photo credit: This Public Domain media comes from the Centers for Disease Control and Prevention's Public Health Image Library (PHIL), with identification number #6806.

If both these factors are mainly influenced by adequate vaccination and not by genetics, frailty or other host factors and BCG has beneficial NSEs, infants that develop a BCG scar and/or a positive TST response following vaccination should have a lower subsequent all-cause mortality risk than infants that do not develop such responses. The first study to test this hypothesis was conducted as part of an MV trial for which children were recruited at six months of age.[5] The study included 1,813 BCG-vaccinated infants examined for BCG scar status and an additional 813 BCG-vaccinated children that were tested for delayed-type hypersensitivity to TST, tetanus, and diphtheria. A total of 92% (1,676/1,813) of infants had a BCG scar, and the scar+/scar- MRR in the first 12 months of follow-up was 0.41 (0.25-0.67); 59% (479/813) of infants had a positive TST and the TST+/TST- MRR was 0.45 (0.24-0.85).[5] Excluding infants that had died of HIV and censoring for TB exposure at home did not change the estimates substantially. There was no difference in the mortality ratio for children that were positive to tetanus or diphtheria skin tests versus those that were negative, indicating that the effect of BCG vaccination was likely due to beneficial non-specific immune stimulation induced by BCG rather than host factors.[5] An additional study with a similar methodology reported a scar+/scar- MRR of 0.45 (0.21-0.96) and evaluated the cause of death in these two early cohorts; having a BCG scar significantly reduced the risk of dying from malaria, the MRR being 0.32 (0.13-0.76).[132]

These early findings sparked routine monitoring of TSTs and BCG scarring in a birth cohort in Bissau, and the findings were reproduced. In a large cohort of infants evaluated for TSTs and BCG scars at two months of age (n=2,332) or six months (n=1,817), having a TST response at two or six months of age was associated with a reduction in subsequent mortality up to 18

months of age, the MRR being 0.54 (0.30-0.99). Within the same cohort, having a BCG scar versus no scar was associated with an MRR of 0.55 (0.31-0.96).[6] Three additional observational studies from Bissau have corroborated these findings. Importantly, the beneficial effect of having a BCG scar among BCG-vaccinated infants is consistent across studies with varying prevalence of BCG scarring (from 52% to 93%). This makes selection bias, hereditary conditions or host factors (e.g., that the most frail infants do not respond with a scar) less likely explanations to the findings.[7,133,134] A review of the studies (summarized in Figure 13) that has evaluated the association between BCG scarring and all-cause mortality and embodied prospective follow-up has recently been conducted.[4]

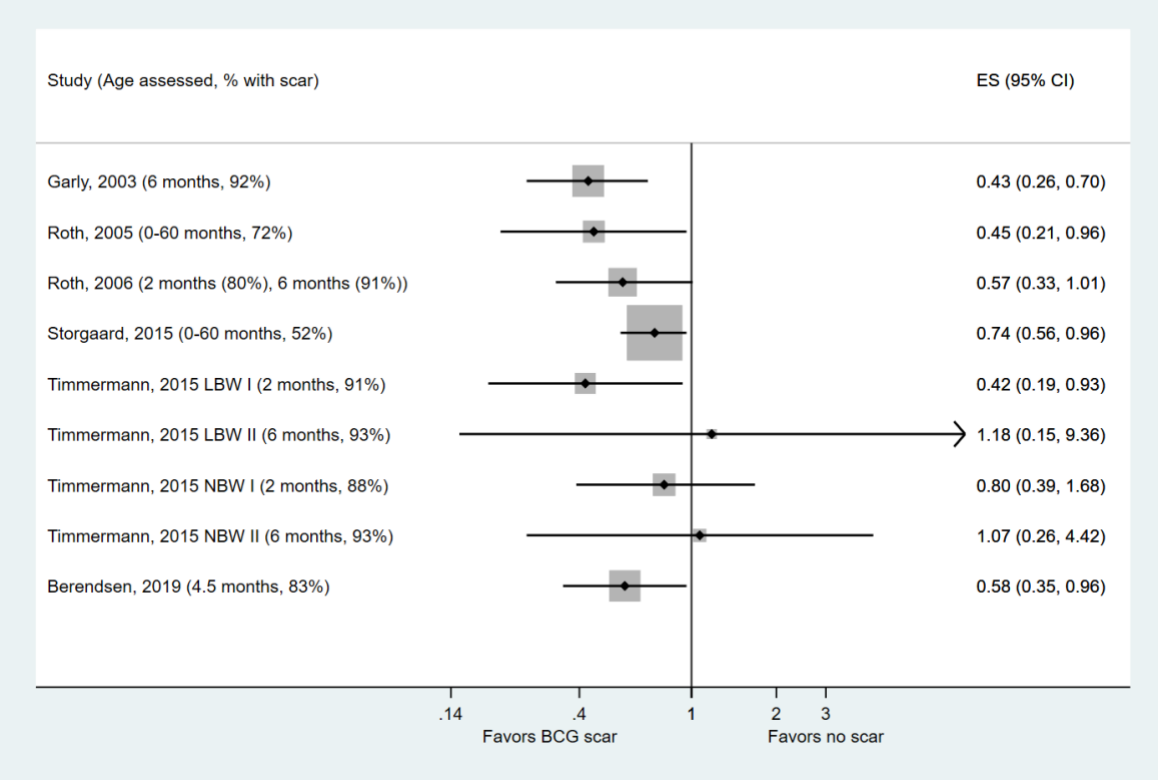


Figure 13. Forest plot of studies that have evaluated the effect on all-cause mortality of having a BCG scar versus no scar among BCG-vaccinated infants.

Abbreviations: LBW, low birth weight; NBW, normal birthweight.

In the just-published meta-analysis of the studies shown above, having a BCG scar versus no BCG scar was associated with an MRR of 0.61 (0.51-0.74).[4] The beneficial effect of having a BCG scar was strongest during the first year, MRR=0.48 (0.37-0.62), and in the neonatal period, MRR=0.45 (0.36-0.55). Three studies have evaluated the effect of having a TST response (yes/no) on the subsequent all-cause mortality in four cohorts of BCG-vaccinated infants (Table 8, Figure 14).

Table 8. Studies reporting prospective all-cause mortality by TST Reaction Status.

Study	Age at TST reaction assessment	Prevalence of TST Reactions	Period of Follow-up	Reactor/nonreactor aMRR (95% CI)
Vaccine 2003[5]	7.5 months	59% (479/813)	7.5-19.5 months	0.48 (0.25-0.90)
Roth 2006[6]	2 & 6 months	30% (465/1,566)	2-18 months	0.54 (0.29-0.99)
Timmermann 2015 LBW[7]	2 months	17% (134/807)	2-12 months	0.47 (0.14-1.54)
Timmermann 2015 NBW[7]	2 months	36% (598/1,663)	2-12 months	0.75 (0.40-1.42)
Timmermann 2015 LBW	6 months	28% (173/621)	6-12 months	0.52 (0.12-2.33)
Timmermann 2015 NBW	6 months	35% (477/1,350)	6-12 months	1.13 (0.45-2.86)

Abbreviations: LBW, low birth weight; NBW, normal birthweight.

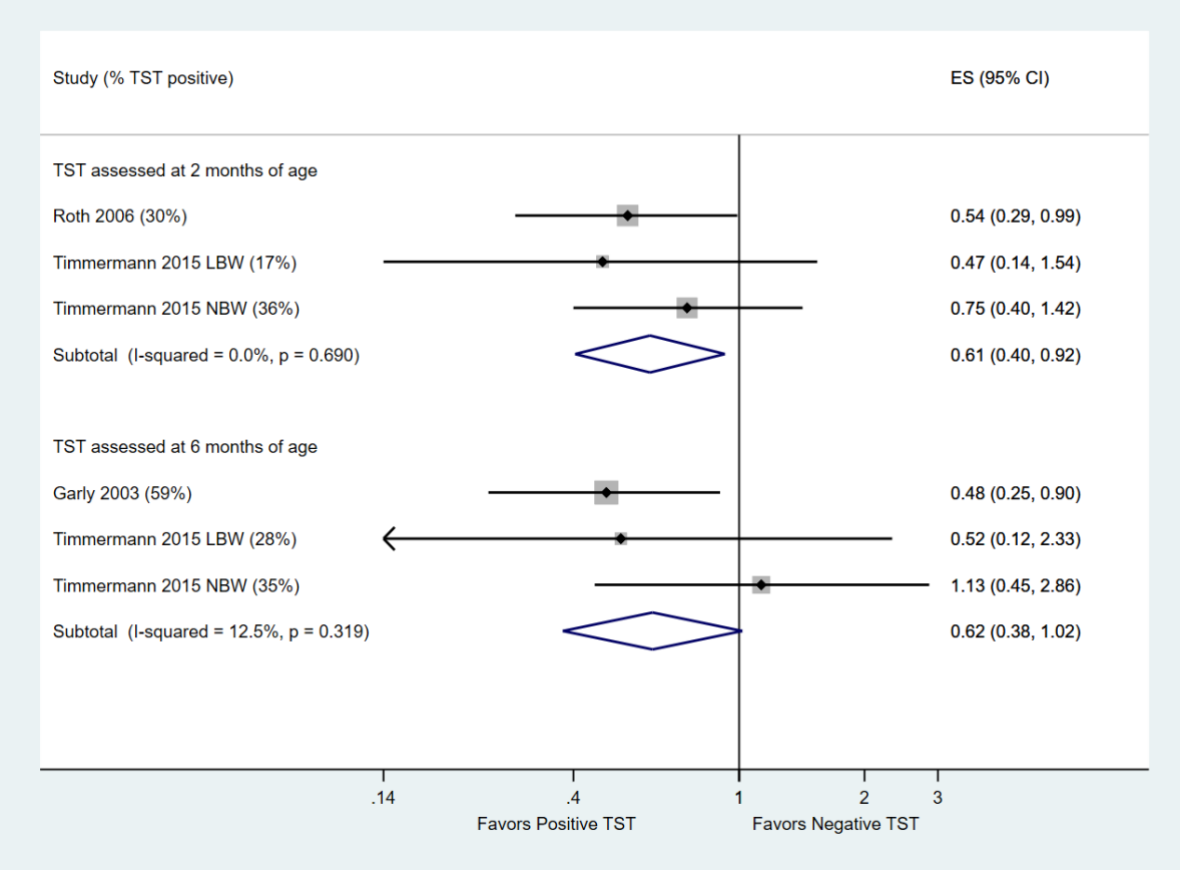


Figure 14. Forest plot of studies reporting prospective all-cause mortality by TST reaction status at 2- and 6-months of age.

Abbreviations: LBW, low birth weight; NBW, normal birthweight. Note: Roth et al[6] included infants who had their TST assessed at both two and six months of age. Infants with a negative TST at two months who had a positive TST at six months were categorized as TST reactors. We are currently awaiting data from the first author separated by the time of assessment (two or six months) for the analysis in paper V.

In the above data, the prevalence of TST reactions varied between 17% to 36% at two months of age and from 28% to 59% at six months of age. Significant associations with mortality were both identified in studies with low (Roth 2006, 30%) and high (Garly 2003,

59%) prevalence of TST reactions, indicating that the findings were not simply due to healthier infants responding with a positive TST response.

As mentioned, the dose of BCG provided and thus the number of culturable particles in the BCG preparation, are the main determinants for developing a TST response and a BCG scar. A study has evaluated 11 different BCG strains grown in the same laboratory at Statens Serum Institut in Copenhagen; the strains were administered to Danish and Indian infants.[135] The study concluded that there were significant strain differences and that the TST response depends on the dose of BCG or "*in a quantitative sense on the strength of the vaccine*".[135]

Interestingly, the latest scar study conducted in Bissau revealed that having a BCG scar is more beneficial if the infant's mother also has a BCG scar. In a cohort of 2,213 BCG-vaccinated infants enrolled in an MV trial at age 4.5 months, 83% of the infants had a BCG scar which was associated with a 41% (5% to 64%) lower mortality between 4.5 to 36 months compared to no scar.[134] But the reduction was 66% (33% to 83%) if the mother also had a scar and only 8% (-83% to 53%) if the mother had no scar (test for interaction, p=0.04).[134] If maternal BCG scarring affects the immune response in the offspring, it has profound implications for both our basic understanding of the immune system and BCG vaccination and its application, both in terms of protection against TB and NSEs. An elaboration on the potential role of maternal immune priming can be read in the Appendix section 8.2.

2.10.2 Randomized controlled trials evaluating the effect of BCG on mortality from causes other than TB

Five historical trials have evaluated the effect of BCG on mortality with separate reporting of deaths caused by causes other than TB.[107] One trial from the UK evaluated both BCG-Denmark and Vole bacillus vaccine.[18] Additionally, five RCTs have been conducted more recently with main outcomes being either all-cause neonatal, infant or in-hospital mortality; endpoints that are unlikely to be influenced by the prevention of TB. The trials are summarized in Table 9 (adapted after Shann[107]).

Table 9. Trials of the effect of BCG or vole bacillus vaccine on mortality from causes other than TB.

Study	Year published	Location	Age followed	Allocation	BCG strain used	Reduction in mortality (95% CI)
Historic trials reporting the effect of BCG on mortality from causes other than TB						
Levine[136]	1948	USA	0-16 years	Alternate	BCG-Phipps	48% (-4% to 75%)
Aronson[137]	1948	USA	0-20 years	Alternate	BCG-Phipps	19% (-21% to 46%)
Ferguson[138]	1949	Canada	0-15 years	Random	BCG-Frappier	12% (-33% to 42%)
MRC[18]	1959	UK	14-21 years	Odd/even	BCG-Denmark	53% (-12% to 83%)
MRC[18]	1959	UK	14-21 years	Odd/even	Vole bacillus vaccine	32% (-98% to 78%)
Rosenthal[139]	1961	USA	0-13 years	Alternate	BCG-Tice	-4% (-682% to 86%)
Meta-analysis estimate by Professor Frank Shann of the effect of BCG or vole bacillus vaccine on non-TB mortality in the historic studies[107]						25% (6% to 41%)
Recent RCTs of BCG provided at birth – effects on all-cause mortality.						
Aaby[140]	2011	Guinea-Bissau	0-28 days	Random	BCG-Denmark+OPV	45% (11% to 66%)
Biering-Sørensen[141]	2012	Guinea-Bissau	0-28 days	Random	BCG-Denmark+OPV	72% (-37% to 94%)
Biering-Sørensen[8]	2017	Guinea-Bissau	0-28 days	Random	BCG-Denmark+OPV	30% (-4% to 53%)
Jayaraman I[48]	2019	India	0-28 days	Random	BCG-Russia	5% (-13% to 20%)
Jayaraman II[48]	2019	India	0-28 days	Random	BCG-Russia+OPV	-1% (-23% to 17%)
Meta-analysis estimate of recent RCTs (Fixed effects)						9% (-2% to 19%)

Aside from the trials referenced above, an RCT of BCG versus no BCG (placebo) among American Indians and Alaska Natives has also reported deaths for both TB and other causes.[17] These data are discussed in detail below (Section 2.10.5).

2.10.3 Effects of BCG on the risk of fatal infection

Within the RCTs of BCG versus no BCG to LBW neonates, the trial reports indicated that the main effect of early vaccination was a reduction in the risk of fatal sepsis. The data outlined in Table 10 indicate, however, that the beneficial effects of BCG were not limited to affecting the risk of neonatal sepsis; the effect against neonatal sepsis versus other causes seems to have been similar (p for same effect=0.92). Several of those deaths were, however, deemed as caused by infectious diseases (which might have indeed progressed to sepsis before the death of the infant, but were nevertheless not categorized as such). The data might reflect limitations in the accuracy of diagnoses collected by verbal autopsy in a setting with limited diagnostic possibilities, or that BCG protects against a wide range of infections.

Table 10. RCTs of BCG versus no BCG to LBW neonates conducted in Guinea-Bissau, neonatal mortality due to sepsis and other causes (verbal autopsy data).

Study	Neonatal sepsis: deaths/included		Risk Ratio (Fisher's 2-sided exact test)	Other causes: deaths/included[a]		Risk Ratio (Fisher's 2-sided exact test)
	BCG	Control		BCG	Control	
Aaby 2011[140]	13/1,168	22/1,152	0.58 (0.30-1.15)	14/1,168	26/1,152	0.53 (0.28-1.01)
Biering-Sørensen 2012[141]	2/50	3/54	0.72 (0.13-4.13)	0/50	3/54	-
Biering-Sørensen 2017[8]	21/2,059	33/2,061	0.64 (0.37-1.10)	23/2,059	29/2,061	0.79 (0.46-1.37)
Total[b]	36/3,277	58/3,267	0.62 (0.41-0.94)	37/3,277	58/3,267	0.64 (0.42-0.96)

[a]Includes unknown causes of death. [b]Test of heterogeneity (neonatal sepsis deaths vs. other causes): p=0.92.

When stratified by infectious/non-infectious causes, the infectious disease estimates are separately significant for the two large RCTs. In contrast, the estimate for non-infectious diseases fails to reach significance (p for same effect=0.30, Table 11). BCG thus appears to have exerted a substantial part of its beneficial effects on the overall risk of dying of infection in both of the large-scale RCTs. Still, an effect on other conditions cannot be ruled out with the available data.

Table 11. RCTs of BCG versus no BCG to LBW neonates conducted in Guinea-Bissau, neonatal mortality due to infectious diseases and non-infectious conditions (verbal autopsy data).

Study	Infectious diseases deaths/included		Risk Ratio (Fisher's 2-sided exact test)	Non-infectious conditions[a] deaths/included		Risk Ratio (Fisher's 2-sided exact test)
	BCG	Control		BCG	Control	
Aaby 2011[140]	20/1,168	34/1,152	0.58 (0.34-1.00)	6/1,168	11/1,152	0.54 (0.20-1.45)
Biering-Sørensen 2012[141]	2/50	3/54	0.72 (0.13-4.13)	0/50	3/54	-
Biering-Sørensen 2017[8]	25/2,059	44/2,061	0.57 (0.35-0.93)	17/2,059	14/2,061	1.22 (0.60-2.46)
Total[b]	47/3,277	81/3,267	0.58 (0.41-0.83)	23/3,277	28/3,267	0.82 (0.47-1.42)

[a]Sudden infant death syndrome, respiratory distress syndrome, anemia, congenital disorders, prematurity, bleeding, and other noninfectious conditions. [b]Test of heterogeneity (infectious disease deaths vs. non-infectious conditions): p=0.30.

In one of the BCG scar studies, death causes were evaluated based on verbal autopsy data. As mentioned, the study reported that having a BCG scar significantly reduced the risk of dying of malaria when compared to children having no scar, the MR being 0.32 (0.13-0.76).[132] Interestingly, a recent observational study that evaluated the effects of BCG on neonatal mortality revealed that BCG was particularly beneficial when administered from November to January, coinciding with the seasonal peak in malaria infections.[142] The BCG/control HR was thus 0.41 (0.25-0.66) in the high malaria transmission period (September-January) and 0.83 (0.56-1.23) in the low malaria transmission period (February-August), p=0.02 for interaction between "malaria season" and BCG.[142]

A recent observational study that included 34,206 children in thirteen Sub-Saharan countries reported that BCG vaccination was associated with a reduced malaria prevalence (adjusted OR 0.94 (0.90-0.98)).[143] The association was stronger for children where the BCG vaccination status was derived from vaccination card data only (e.g., excluding

maternal recall), aOR 0.88 (0.82-0.94) and in areas with suboptimal BCG coverage, aOR 0.81 (0.73-0.89).[143] These intriguing indications of beneficial effects of BCG on malaria risk corroborate reports indicating that BCG protects against malarial parasitemia both in mice and in humans.[144,145]

BCG has also been shown to protect against experimental infection with an attenuated yellow fever virus vaccine strain in a randomized placebo-controlled human challenge study[146] and against disseminated candidiasis in mice with severe combined immunodeficiency.[9] BCG's beneficial immune stimulus thus appears to induce protection against a broad range of infectious agents.

2.10.4 Potential immunological mechanisms

At least two mechanisms may explain the beneficial NSEs of BCG on all-cause mortality: immediate effects on the granulopoietic capacity mediated by induction of G-CSF, and trained immunity. In brief, BCG's effects on the granulopoietic capacity through induction of G-CSF was recently shown in mice. BCG fuelled an emergency granulopoiesis response leading to a dramatic increase in the neutrophil count.[147] Higher neutrophil numbers were then directly and quantitatively responsible for drastically improved survival among BCG-vaccinated mice versus controls in a murine model of neonatal polymicrobial sepsis.

In a landmark study, trained immunity was demonstrated in humans for the first time, and it was induced by BCG.[9] When BCG was provided to healthy volunteers, there was a four- to sevenfold increase in their IFN-γ production and a twofold enhanced release of monocyte-derived cytokines in response to unrelated bacterial and fungal pathogens. This enhanced function of monocytes was present for at least three months after vaccination and was induced through the NOD2 receptor.

For further information on these two potential immunological mechanisms, please see sections 8.3.1 and 8.3.2 in the Appendix.

2.10.5 Long-term effects of BCG vaccination and effects on diseases other than TB

In Denmark, a large case-cohort study evaluated the effects of BCG and/or vaccinia (smallpox) vaccination among 46,239 Copenhagen schoolchildren born between 1965-76 with follow-up until 2010. Vaccination with BCG was associated with an aMRR of 0.58 (0.39-0.85) for natural death causes (includes cancers, cardiovascular diseases, infectious diseases, neurological disease, autoimmune diseases, and other diseases).[148] As a control outcome, the effect of vaccination towards deaths due to accidents, suicide or murder was evaluated. For BCG versus no BCG, the combined aMRR for these causes was 0.87 (0.55-1.38).[148]

In an RCT with 60 years of follow-up, Aronson et al have reported the effects of BCG versus placebo among American Indians and Alaskan Natives that were recruited between 1935-1938.[17] The study reported that BCG vaccine efficacy persisted for 50-60 years with a slight waning in efficacy over time. Interestingly, by the 1st January 1948, there had been 56 deaths (nine TB, 46 other causes, one unknown cause) among 1,540 BCG-recipients versus 114 deaths (55 TB, 58 other causes, one unknown cause) among 1,423 placebo-

recipients.[17] The mortality rate from causes other than TB was thus 3.0% (46/1,540) in the BCG group and 4.1% (58/1,423) in the placebo group, the BCG/placebo RR being 0.73 (0.50-1.07) (2-sided Fisher's exact p=0.11). In the subsequent long-term analysis, there were 1,483 in the BCG group and 1,309 in the placebo group. Aside from a higher TB incidence in the placebo group (63 cases compared to 27 in the BCG group), there was also a higher diabetes mellitus prevalence for placebo-recipients versus BCG-recipients (26% versus 22%, p=0.02). Another randomized study reported that BCG can bring blood sugar levels (hemoglobin A1c) back to near normal levels among type 1 diabetes subjects with long-term disease.[149] This might indicate that BCG not only has a beneficial effect on the innate immune system but that it also reduces inflammation in the steady-state. Such a hypothesis finds support in reports that unstimulated blood samples have lower overall cytokine concentrations (mainly for IL-10, MIF, IP-10, and IL-8) in BCG-vaccinated infants versus BCG-naïve infants.[150]

The authors also reported the incidence of malignancies (BCG 11%, placebo 13%, p=0.13) and renal failure (BCG 6%, placebo 7%, p=0.09). In terms of preventing death, the death rate was 935/100,000 person-years in the BCG group and 958/100,000 in the placebo group, the vaccine efficacy to prevent deaths from causes other than TB being 2% (-11% to 14%).[17] In a recent report from the same study, the authors reported the total all-cause mortality figures, and they were 41% (632/1,540) in the BCG group versus 44% (633/1,423) in the placebo group.[151] The corresponding all-cause mortality RR for BCG versus placebo is thus 0.93 (0.85-1.01) (2-sided Fisher's exact p=0.07). The study reported an overall cancer rate that was not significantly different for BCG vaccine versus placebo recipients, the HR being 0.82 (0.66-1.02). The rate of lung cancer was significantly lower in BCG (18 cases per 100,000 person-years) versus placebo recipients (45 cases per 100,000 person-years), the hazard ratio being 0.38 (0.20-0.74).[151] The mortality rate from lung cancer was also reduced in the BCG group (13 deaths per 100,000 person-years) versus 41 deaths per 100,000 person-years in placebo recipients, the HR being 0.32 (0.15-0.68).[151]

A recent study has investigated whether the immune modulation associated with BCG vaccination might reduce the risk of Alzheimer's disease. In a group of bladder cancer patients, 64% (878/1,371) received post-operative intravesical treatment with the BCG-oncoTice strain. During a median postoperative follow-up period of eight years, the risk of Alzheimer's disease was 2.4% (21/878) in the group treated with BCG versus 8.9% (44/493) for those not treated with BCG, the no BCG/BCG HR being 4.78 (2.84-8.05).[152]

3 Hypotheses and aims

The present Ph.D. thesis aimed to investigate the following three hypotheses:

Hypothesis A. *BCG vaccines possess beneficial, non-specific effects related to mortality and morbidity. Since BCG strains are phenotypically distinct, the size of these effects might be different. Compared with BCG-Russia, vaccination with BCG-Denmark and BCG-Japan is associated with fewer admissions, lower in-hospital case-fatality and fewer deaths.*

Hypothesis B. *The proportion of children having a BCG scar and a positive PPD response is 30% lower among children vaccinated with BCG-Russia compared with BCG-Denmark and BCG-Japan.*

Hypothesis C. *BCG vaccines are renowned for their safety. Since BCG-Denmark and BCG-Japan are probably more potent vaccines, they might be associated with a higher rate of adverse events when compared to BCG-Russia.*

Five RCTs conducted in Guinea-Bissau between 2002-2018 have provided data to test these hypotheses; the Ph.D. student supervised data collection procedures for three of these RCTs during a research year (2012, RCT III) and the Ph.D. period (2015-2018, RCT IV-V). The RCTs are listed in chronologic order below with a designation of the specific hypothesis addressed and the corresponding Ph.D. paper to which the RCT contributed data.

3.1 RCTs of early BCG-Denmark versus no BCG among low birth weight infants

RCTs I-III conducted from 2002-14, hypothesis A, papers II, IV & V.

Based on the aforementioned observational studies that indicated a beneficial effect of BCG on all-cause infant mortality, there was a need for an RCT to test whether BCG could reduce mortality. The official policy at the time was to postpone BCG vaccination for LBW children in Guinea-Bissau. This meant that a group of neonates that would normally not receive BCG and that had high mortality due to their low birth weight could be studied. The first RCT, which was to test the hypothesis that early BCG versus no BCG provided to LBW neonates is associated with a 25% reduction in infant mortality, was initiated in November 2002.[140] The study was the first RCT to be conducted at the HNSM Maternity Ward. There were, unfortunately, problems with the randomization procedures during parts of 2003 and 2004, which meant that the first 1,309 children had to be excluded.[140] Since randomization procedures at the HDSS health centers had been sufficient, the effects of BCG in a small cohort of 104 infants could be reported.[141] After an examination of all study procedures, the main trial was reinitiated and conducted between 2004-08.

The sample size of 1,600 infants for the main trial was calculated based on a pretrial (1990-98) infant mortality of 250 per 1,000 for LBW infants born at HNSM.[140] Simultaneously, a vitamin A trial with a sample size of 1,600 children was conducted; the two trials were conducted as a 2-by-2 factorial trial. Given that there was no interaction between BCG and vitamin A, and since both trials maintained the same randomization to BCG, the effect of BCG on infant mortality was analyzed in a combined dataset comprising 2,320 infants. The primary outcome was infant mortality, and secondary outcomes were adverse events assessed three days after inclusion, whether the effects of BCG were modified by TB-

exposure and the impact of early BCG on vaccination coverage. Other outcomes were effects on growth, BCG response (TST and BCG skin reactions), and hospital admissions.

The secondary outcome hospital admissions was later reported as a combined analysis covering all three RCTs of BCG to LBW infants from Bissau in paper II. The a priori hypothesis for that project was that early BCG would be associated with a 50% reduction in the risk of hospital admission during the neonatal period.

The accumulated infant mortality among LBW controls was 101/1,000, e.g., less than half the level observed before the trial started, and the effect of BCG versus no BCG on infant mortality was an MRR of 0.83 (0.63-1.08). The authors reported the effect of BCG on all-cause mortality also by the time of the home follow-up visits, which were conducted three days, 28 days, two and six months of age. By 28 days (before the infants had received other vaccines), the MRR for BCG versus no BCG was 0.55 (0.34-0.89).[140]

Due to the vast implications of this finding, a confirmatory trial to test effects of early BCG was conducted.[8] Initially, the sample size for this trial was 3,050 infants, based on the control group neonatal mortality rate of 4.2% in the 2004-08 trial and that BCG would be associated with a 45% reduction in all-cause neonatal mortality. Since the neonatal mortality further decreased to 3.2% during the trial, the sample size was increased to 4,100 infants. The trial reported a 30% reduction in neonatal mortality associated with BCG, the MRR for early BCG versus control being 0.70 (0.47-1.04).[8] The meta-analysis of the three RCTs indicated substantial reductions in mortality associated with early BCG vaccination when assessed both at three days, 28 days and twelve months after birth.[8]

3.2 A randomized trial of providing BCG vaccination immediately to neonates admitted to the intensive care unit in Guinea-Bissau: Effect on mortality

BCGIMED trial (RCT IV) conducted from 2013-2018, hypothesis A, papers I & IV.

The BCGIMED trial was planned to investigate whether providing BCG immediately at birth versus at discharge could lower in-hospital mortality among neonates admitted to the nursery at the HNSM Maternity Ward.[123] The trial thus made use of the fact that such infants would normally only receive BCG and OPV at discharge from the hospital. Before the trial was commenced, the mortality among infants admitted to the nursery (which features very basic equipment such as incubators, oxygen and occasional provision of glucose and/or milk formula) was 14% (254/1,877). Neonates were admitted for an average of five to six days (Study Protocol, Appendix section 0). Based on the results of RCT I and II, the a priori hypothesis was that immediate BCG would be associated with a 40% reduction in the in-hospital mortality when compared to controls (primary outcome), and secondary outcomes were the cause of death and admission length. The randomization procedures were changed during the trial to include stratification by sex and weight group, after consultation with the DSMB. Additional exclusion criteria were also added: inclusion weight<1,250 g, 1-minute Apgar score of <2, and gross malformation since the mortality risk was very dependent on these factors. For example, the mortality for Maternity Ward neonates weighing <1,000 g was 82% (91/111) between 2007-13, whereas it was 2.5% (820/32,928) for infants weighing>2,000 g (Study Protocol, Appendix section 0). The exclusion criteria and

stratification by weight group was thus introduced to prevent that very LBW neonates might skew the results of the trial by chance. In April 2015, an immunological sub-study nested within BCGIMED was initiated through an international research collaboration to identify molecular signatures of survival induced by BCG in a systems biology approach. To this end, provided that separate informed consent had been obtained, BCGIMED neonates were bled one day after vaccination with BCG+OPV versus no vaccination (control). The blood samples are currently being processed by our collaborators in Vancouver, Canada, for epigenomic and transcriptomic signatures associated with BCG vaccination. A paper detailing initial results (described above) is currently under review. The original sample size of the BCGIMED study was 1,262 infants based on an expected in-hospital mortality of 12% to detect at 40% difference in in-hospital mortality between BCG-vaccinated and BCG-unvaccinated neonates (based on 123 events) with 80% power and a significance level of 5% (Study Protocol, Appendix section 0). A long-term trend of declining mortality and the establishment of a new neonatal intensive care unit by MSF meant that the trial was discontinued after 3,353 inclusions and 107 events in August 2017, after consultation with the DSMB. Within the trial, infants randomized to the control group received BCG and OPV at discharge from the Maternity Ward. All infants that resided within Greater Bissau were transported home and received standardized follow-up visits conducted at three days, two months, six months and twelve months. This was mainly done as a precaution to monitor adverse events related to immediate BCG vaccination of frail infants. Since data on BCG reactions and mortality was also collected at the visits, the study provided data regarding the NSEs of BCG among infants that develop a local BCG skin reaction versus no reaction (paper IV) and additional papers based on data from the study are planned.

3.3 Evaluating the effectiveness of different BCG strains in Guinea-Bissau: A randomized trial of the impact on neonatal hospital admissions.

BCGSTRAIN I study (RCT V), 2014-2018, hypotheses A-C, papers I, III, IV, and V.

The main hypothesis of this trial was that BCG strains are not equal due to genetic differences and manufacturing differences. The hypotheses were based on observational data from Uganda indicating significant differences in mycobacteria-specific and non-specific immune responses and BCG scar rates[20], a retrospective study from Kazakhstan indicating that BCG-Japan might better prevent TB than BCG-Russia[22], and the RCTs from Guinea-Bissau indicating substantial NSEs associated with BCG-Denmark.

We hypothesized that:

1) BCG-Denmark would be associated with 30% fewer hospital admissions than BCG-Russia both in the neonatal period and in the first six weeks of life, and

2) the proportion of infants having a BCG scar and a positive TST response would be 30% lower among infants that received BCG-Russia compared with receiving BCG-Denmark.

The primary outcome of the trial was hospital admissions at the HNSM Pediatric Ward occuring within the first six weeks of life. Mortality in the same period was a secondary

outcome along with BCG scar frequencies at two and six months of age, TST response at six months of age and adverse events (lymphadenitis).

Healthy infants could be included at the time of discharge from the HNSM Maternity Ward if the infant had been born at HNSM with no severe malformations. Informed consent to participate in the study was sought from the mother/guardian. The trial applied several novel procedures. It was the first BHP trial to apply follow-up by telephone (an analysis of which will be published separately) and therefore the first study that could recruit all infants that had been born at the ward, regardless of the area of residence. The sample size of 12,000 infants (6,000 to receive BCG-Denmark and 6,000 to receive BCG-Russia) was based on the assumption that 2.5% would be hospitalized by six weeks of age, providing 80% power to detect a 30% difference in hospitalization frequency with an alpha of 0.05. Since the pretrial mortality risk from birth to 6 weeks of age was also 2.5%, the trial would potentially have power to show a 30% reduction in mortality (secondary outcome) as well. Approximately halfway into the trial, the intervention (BCG-Denmark) became unavailable due to a production halt. After counseling with the Data Safety Monitoring Board which recommended to continue the study using BCG-Japan, the study group applied the Guinean and Danish Ethical Committees for permission to exchange BCG-Denmark with BCG-Japan and finish the study as a natural experiment. While the study thus had reduced power for the main comparisons of morbidity and mortality associated with the different strains, the circumstances did provide us with an important possibility to evaluate the BCG-Japan vaccine – a WHO-prequalified BCG vaccine that has generally been held at high esteem worldwide.

4 Materials and methods

4.1 Setting

4.1.1 The Bandim Health Project research station in Guinea-Bissau

As described in detail earlier, Bandim Health Project was established in Guinea-Bissau in 1978 to evaluate (in a one-year study) the reasons behind a very high mortality for children <5 years of age observed in the years following Guinea-Bissau's independence from Portugal. Guinea-Bissau is located close to the Equator at a latitude of 11°, and 55% of 2-15-year old children in the capital Bissau have gastrointestinal parasites.[153] Most roads are dirt roads (Figure 15), houses are built rather close to each other, and many in the capital keep chickens, goats, and pigs within the urban environment.

Figure 15. Two children in BHP's urban HDSS in Bissau, Guinea-Bissau. Photo credit: Sofia

BHP was named after the neighborhood where the first census was made, Bandim, where the BHP's offices are also located today (Figure 16).

Figure 16. The BHP data entry building in Bandim, Bissau. Photo credit: Bandim Health Project.

Since then, BHP has evolved into a multifaceted research station focusing on the evaluation of early-life interventions (mainly vaccines and vitamin supplementation) with research branches also in HIV, TB, and malaria.

Since Peter Aaby conducted the first census of the population in the *Bandim* neighborhood, the urban HDSS maintained by BHP has gradually expanded to include a larger part of Bandim and the Belém neighborhood (1984), Mindará (1994) and the Cuntum 1 and Cuntum 2 neighborhoods (2002). The population included in the urban HDSS (Figure 17) is slightly more than 100,000 individuals and a similar population is followed in a rural HDSS, which was established in 1990 and now includes 222 randomly selected village clusters across Guinea-Bissau (Figure 18, each red dot represents a rural HDSS cluster, and the larger red dot represents the capital, Bissau).[154]

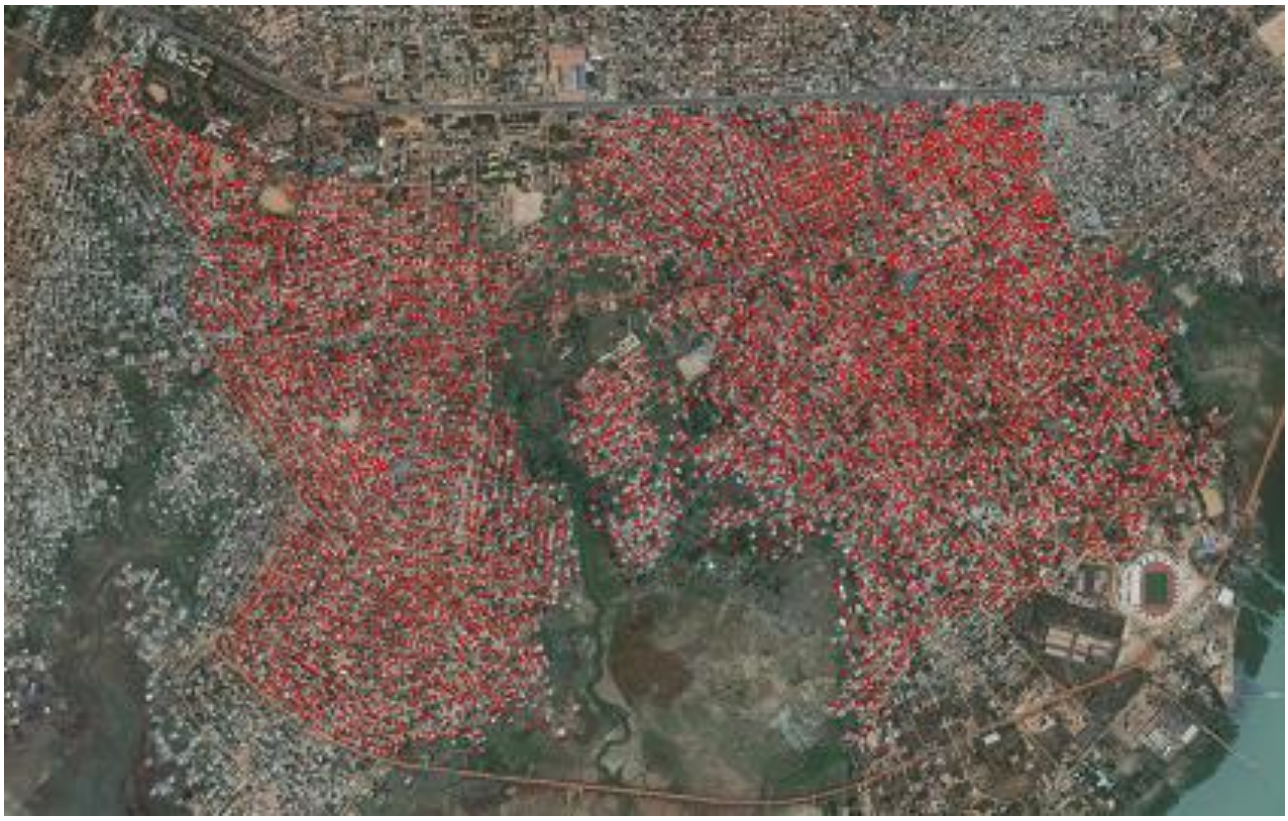


Figure 17. Urban BHP HDSS study area (each red dot represents a HDSS house). The GPS-tagged figures detailing the urban and rural HDSS were kindly provided by Andreas Rieckmann.

Guinea-Bissau has suffered from chronic political instability, including a damaging civil war from 1998 to 1999 and several coup d'états. The country is among the world's poorest, and child mortality is among the world's highest.[155] The health care system is strained and affected by a lack of resources and a lack of doctors, including a severe lack of specialized senior doctors.

BHP collaborates closely with the Ministry of Health and the Institute of Public Health in Guinea-Bissau. As an example of the results of this collaboration, the official BCG vaccination policy was expanded to include all newborns regardless of birth weight, due to the findings in RCT I-III.

Figure 18. Rural BHP village clusters (red dots, the larger dot is the capital, Bissau).

4.1.2 A platform to evaluate real-life effects of health interventions provided at birth: Collaboration between BHP and Hospital Nacional Simão Mendes

Papers I-V

Figure 19. The Maternity Ward at HNSM. Photo: The author.

Established in 2002, the collaboration between the BHP and HNSM's Maternity Ward (Figure 19) has provided important insights into the effects of several interventions provided at birth: BCG, OPV, vitamin A and different strains of BCG. To date, the collaboration has paved the way for five completed RCTs[8,67,123,140,156] (of which one was a 2-by-2 factorial RCT). A sixth RCT has finished enrolling[125], and a seventh trial that will also compare different BCG strains is awaiting ethical approval. From 2004-2019, approx. 44,500 neonates have been enrolled in a BHP Maternity Ward RCT, corresponding to >2% of Guinea-Bissau's approximately 2 mill. inhabitants.[157] The collaboration and the resultant studies have not only provided important insights into the NSEs of early health interventions. They have also helped ensure that OPV and BCG was provided to vulnerable neonates at the Ward that otherwise might not have received the vaccines early. For LBW neonates discharged from HNSM between 2002-14, half of the neonates (those randomized to receive BCG) would otherwise not have received BCG, had it not been for the RCTs conducted by BHP. As mentioned, the trials also meant that National guidelines were changed so that all newborns, regardless of birth weight, should be given BCG. Also, as part of trial procedures, a substantial number of infants and their mothers have been transported home by our team and received follow-up visits at two, six and twelve months of age. All infants accessed for eligibility were offered free medical consultations and certain medications free of charge, regardless of trial participation. The trials have thus, beside of the important results for public health, had a substantial positive impact on its participants and on the community.

The skilled BHP assistants working in collaboration with the Maternity Ward is the unifying platform for all the studies included in the present Ph.D. project. For Paper I, the two vaccinators provided all vaccinations and counted the number of doses they could retract from each vial of BCG-Denmark and BCG-Russia. For Paper II, the majority of infants were enrolled at the Ward. All infants included in Paper III and practically all in papers IV-V were also enrolled at the Ward (Figure 20 A-E).

Figure 20 A-E. Collage of inclusion procedures in the BCGSTRAIN I study. All photos: Sofia Busk.

A: A mother gives her written consent for her newborn to participate in the study to BHP Maternity Ward supervisor Gabriel Marciano Gomes. She is illiterate, so she signs the informed consent form using her fingerprint. **B:** Inclusion interview. **C:** Measurement of the maternal MUAC. **D:** The mother assists in the collection of telephone numbers from stored contacts on her telephone. **E:** Upon visual confirmation of the neonates' sex, the mother randomizes her son after the sex by picking a closed envelope from a stack of male randomization envelopes held by the BHP inclusion supervisor Odete Correia.

Aside from facilitating vaccination at the ward, the BHP has played an important role in the digitalization of birth records. Since 2007, a database of all births has been maintained, and a dedicated fieldworker collects data from all mothers present at the ward, including a supplementary interview with mothers from the HDSS, while a data entry clerk digitalizes the data.

4.1.3 Systematic data collection at the Pediatric Ward of Hospital Nacional Simão Mendes: Surveillance of hospital admissions and in-hospital mortality risk

Papers II-III

In close collaboration with the management board at HNSM's Pediatric Ward, BHP has maintained registration of hospital admissions and consultations since the 1990s.[158] The Ward is adjacent to the urban HDSS, and it is the main pediatric treatment referral center in the country. Many infants included in BHP studies thus use the ward (Figure 21).

Figure 21. Aerial view of the Pediatric Ward at HNSM (lower right building). Photo credit: Sofia Busk.

The data collection at the ward has furthermore facilitated many observational studies evaluating hospital admission patterns and in-hospital case-fatality rates associated with different risk factors and different diseases.[159–166] The BHP has been allowed to maintain an office at the Ward, where our assistants can enter data on admitted infants and hospital charts are stored for future reference in our archives (Figure 22).

Figure 22. Working with the data at the BHP entry office of the Pediatric Ward. Photo credit: Sofia Busk

Aside from the important studies that have been conducted based on the data collection, the setup has enabled the local doctors and nurses to follow-up on patients, and BHP has assisted with basic statistics regarding patients admitted per year by admission cause.

Papers II-III of this thesis are based mainly on the database maintained by the BHP at the Pediatric Ward. The database includes information on the date of admission and discharge, names of the mother and father, their telephone contact information (if available), BHP study ID and HDSS ID number (if applicable), weight at admission, probable and definitive diagnoses (depending on the assessment made by the treating physician) and discharge status (discharged alive or died at the ward). In early 2016, we strengthened the data collection with an increased focus on the daily follow-up of admitted infants. Before this initiative, the final discharge status was mainly based on hospital charts provided by the physicians responsible for the patient's treatment. These charts are not always available, however, and information might thus be lost. In the revised routines, the data supervisor performs daily morning rounds to all Ward beds all days of the week to ensure that admissions occurring during the evening and in the night are registered in the database of admitted patients and to ensure that the vital status is updated daily. A subsequent afternoon round by an assistant captures additional information on infants admitted or discharged during the day. Rather than relying on the hospital charts which are not always available, the main source of information for the most crucial data, whether the child was discharged alive or not, is thus the families present at the ward. For those that left the ward before our assistant arrived, the families in adjacent beds can provide information as to whether an infant was discharged alive or not. In cases where this information cannot be obtained using this approach, the infant's family is telephoned to ask whether the infant was discharged alive or if the infant died during the hospital admission.

In summary, hospital admissions at the Pediatric Ward were a secondary outcome in the three RCTs of BCG versus no BCG to LBW infants (Paper II) and the main outcome in Paper III. For Papers IV-V, the data registration at the Pediatric Ward assisted BHP researchers in confirming information on deaths, including the timing of deaths and death causes.

4.1.4 BHP's Health and Demographic Surveillance System

Papers II, III, IV & V

The HDSS provided the backbone for all thesis papers except paper I. Within the urban HDSS, BHP assistants conduct monthly or bi-monthly house visits to register pregnancies in the population surveyed (Figure 23).

Figure 23. A woman from the HDSS is interviewed by HDSS assistant Gilberto da Silva. Photo credit: Sofia Busk.

Additionally, mothers coming for pregnancy consultations at three HDSS health centers are interviewed to register pregnancies. When a pregnancy is registered, the unborn child is given a unique registration number *in utero,* which becomes the child's unique ID number after birth. The ID number includes two digits indicating the neighborhood and zone of residence of the family and a 5-digit number indicating the unique registration number within the specific zone and neighborhood (Figure 24).

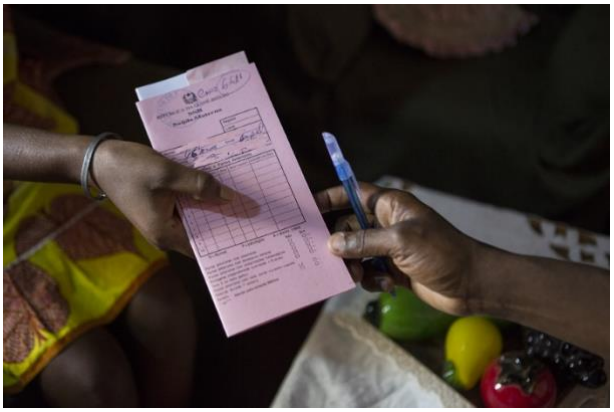


Figure 24. Upon registration of the pregnancy, a pregnancy card is provided to the mother to be used at subsequent pregnancy consultations at nearby health centers. Photo credit: Sofia Busk.

In the five RCTs that provided data included in this thesis, a substantial number of the included infants were from the HDSS: LBW RCT I: 100% (104/104), LBW RCT II: 30% (713/2,320), LBW RCT III: 30% (1,252/4,159), BCGIMED RCT IV: 15% (488/3,353), STRAIN I RCT V: 21% (2,529/12,023). In total, 23% (5,086/21,959) of the combined cohort examined in this dissertation were residents of the HDSS. The HDSS facilitated follow-up due to the elegant address system, which helped locate the infants (Figure 25, Figure 26).

Figure 25. Residents relaxing between the houses in Bissau are approached by our follow-up assistant Besna Nhaga during follow-up procedures. If the infant is not home, then neighbors/relatives provide important information regarding the status of the child. Photo credit: Sofia Busk.

The complete address information, including the neighborhood, zone number, and the house number is painted on the facade of the HDSS houses once every two to three years with the blessing of the local residents (Figure 26).

Figure 26. Weighing of a BCGSTRAIN II child at home-visit by six months of age in the Cuntum 1 neighborhood, zone 2, house number 109. Photo credit: Sofia Busk.

The routine HDSS data collection also helped resolve inconsistencies in the data and provided mortality risk estimates between 6-12 months of age for HDSS infants included in RCT V (Paper IV-V). At the visits, vital parameters such as weight, MUAC, and the temperature is recorded, and data on vaccinations provided after discharged is registered (Figure 27). These data can be used in future observational studies.

Figure 27. Vaccination data is recorded from a child's vaccination card as part of routine study data collection procedures.

4.1.5 Data collection by mobile phone interview

Paper III (RCT V)

Most studies conducted at the Maternity Ward has either made use of the HDSS framework for follow-up measures and/or involved transportation of included neonates to their home to ensure that they could be found again. The STRAIN I study applied a novel approach to data collection in Guinea-Bissau. Given that the study would include a substantial portion of the birth cohort at the Maternity Ward (13 infants per day when the BCGIMED study was enrolling simultaneously and 20 infants per day after the BCGIMED study was discontinued), a new approach had to be taken. In the earlier studies, fewer infants (two to three per day in the LBW RCTs) had been included, and these were either transported home by BHP or were HDSS infants that could be located again using the address information collected at inclusion. In the STRAIN I study such an approach was not feasible since the study was planned to randomize 12,000 infants in 3 years. Follow-up by contacting families by telephone was therefore implemented. There was 65 cellular subscriptions per 100 inhabitants in Guinea-Bissau in 2014 when the trial was initiated and 78 in 2017 when enrollment and follow-up had been completed.[167] At inclusion, our team collected telephone numbers of both the mother, father, relatives, people living in the same house and neighbors to the family, to ensure that it would be possible to contact the family again. Nine percent of the cohort had no telephone number recorded at inclusion and were thus lost to follow-up if the family did not reside in the HDSS, and the infant was not subsequently admitted at the Pediatric Ward.

The overall follow-up success rate at six weeks of age was 80% (9,569/12,023); among infants with one or more telephone numbers registered, it was 87% (9,569/11,024).[168] We further noted that successful follow-up largely depended on the number of telephone numbers collected at inclusion (Figure 28).[168]

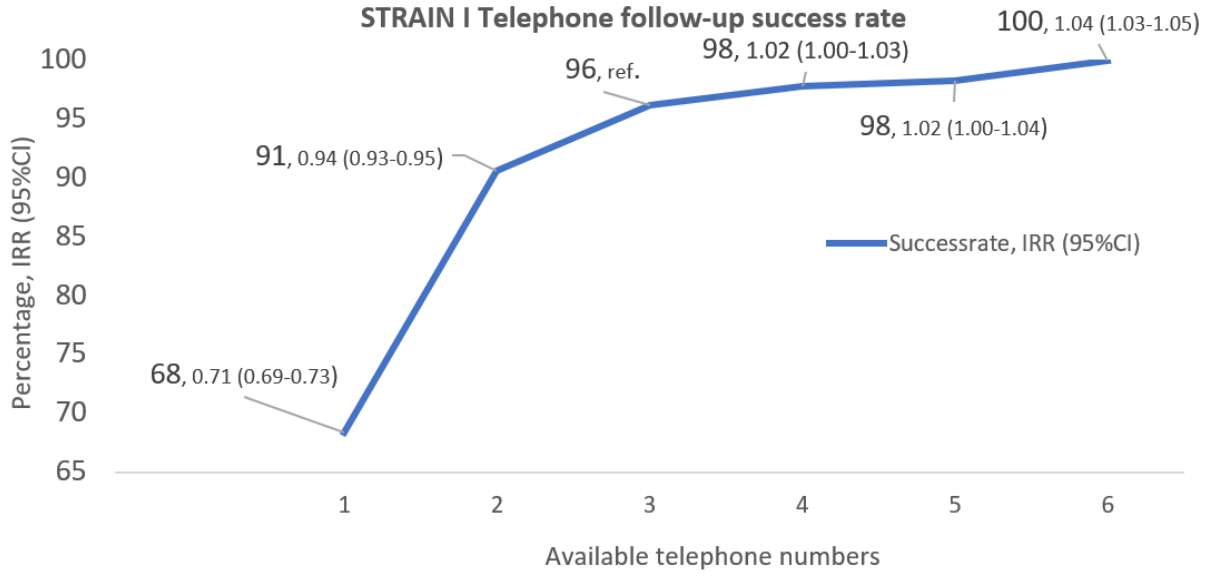


Figure 28. Follow-up success by six weeks of age in percent by available telephone numbers. Kindly provided by Elise Brenno Stjernholm.

For HDSS infants, the success rate of telephone follow-up was 83% versus 96% for home-visits ($p<0.01$ for same rate).[168] A detailed analysis by Stjernholm et al. comparing telephone follow-up data with HDSS data and data from the Pediatric Ward revealed that both home-visits and telephone follow-up are insufficient at capturing information on hospital admissions. Only 30% (38/126) of the admissions identified in the Pediatric Ward database had been reported by telephone and 42% (53/126) at the home-visits.[168] Both approaches were, however, good at capturing information on deaths, 82% (32/39) of HDSS deaths were correctly registered at telephone follow-up, and all unregistered deaths were among infants with unsuccessful follow-up. The same number was 95% (37/39) for home-visits. While preparing the data for the LBW hospital admission study (Paper II), the same pattern regarding reported admissions was noted. There were issues when it was not the mother or father that was interviewed. Also, some reported admissions were regarding infants that had been admitted to the hospital before inclusion. The families would correctly report such an event as a hospital admission, but for trial purposes it had happened before inclusion. The data discrepancies meant that we found the hospital admission data reported by telephone to be unreliable and focused on data collected at the Pediatric ward. This is important since it has implications for studies using maternally reported morbidity as an outcome; the usefulness of such data might be limited, while both telephone follow-up data and home-visit follow-up data was accurate for mortality outcomes. In paper III, we used morbidity data registered at the pediatric ward while mortality data reported by telephone was included in our analyses, consistent with the precision of the collected information detailed in the analysis by Stjernholm et al.[168] Based on the results of the STRAIN I study and the experiences from using mobile phone follow-up in Guinea-Bissau, we initiated another RCT to test BCG-Japan versus BCG-Russia among 15,600 infants.[125] While the trial

also embodies telephone follow-up by six weeks of age as in the STRAIN I trial, we included an additional follow-up call at six months of age to better study the long-term effects of the two BCG strains. Furthermore, we chose to drive families home if ≤1 telephone number was available at inclusion to improve the follow-up success rate.[125] The RCT has finished enrolling, and follow-up procedures for the main outcomes will be completed by September 2020.

4.2 Statistical analyses

4.2.1 Linear regression

In Paper I, we analyzed the number of BCG doses that could be obtained from 20-dose vials, including a comparison between BCG-Denmark and BCG-Russia, using linear regression.

4.2.2 Andersen-Gill Cox regression model for the analysis of recurrent event data

In Papers II and III, we analyzed the risk of hospital admission associated with randomization to BCG versus no BCG and with different BCG strains, respectively. Since infants could have multiple events (hospital admissions), a standard Cox survival model that focuses only on time to the first event would not allow for a complete representation of the collected data. We therefore prepared the data to use the counting process formulation of Andersen and Gill so that an individual could contribute recurrent (multiple) events to the analysis, while not contributing risk-time when admitted.[169] This approach has the basic assumption that all failure types are equal and indistinguishable[170], which was the case for our hospital admission data. Important features of such an approach are that the events are ordered, that the subject can only be at risk of one event at a time, and that risk time is not accumulated during an event.[171] The Andersen-Gill model has been used to evaluate repeated hospitalizations in the elderly and among persons living in low-income areas with low access to healthy food.[172,173] The major limitation of the model is that it does not allow more than one event to occur at a given time. If testing the risk of adverse events associated with a drug and a patient experiences two different adverse events at the same time, it would not be possible to include both observations in the model.[170] Examples of possible trajectories for six study participants analyzed using the Andersen-Gill Cox-model and the corresponding data series are provided in Figure 29 and Figure 30.

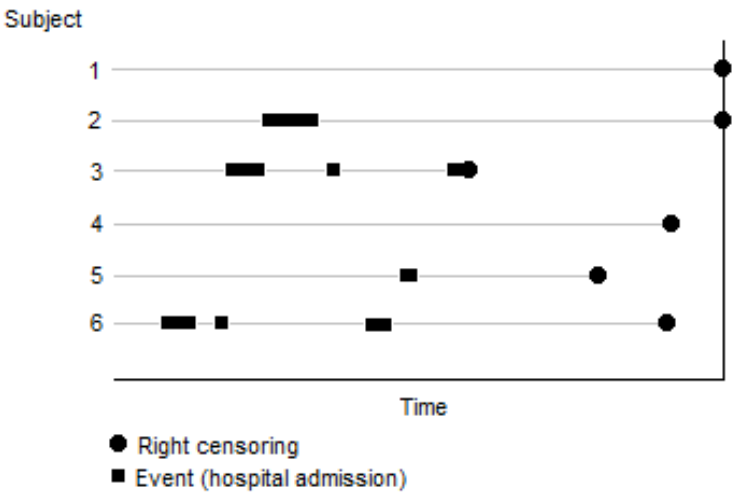


Figure 29. Schematic plot of recurrent time-to-event data for six hypothetical study participants.

id	group	time0	time	admitted
1	BCG	0	42	0
2	Control	0	12	1
2	Control	18	42	0
3	BCG	0	9	1
3	BCG	14	18	1
3	BCG	19	25	1
4	Control	0	39	0
5	BCG	0	23	1
5	BCG	25	37	1
6	Control	0	4	1
6	Control	9	11	1
6	Control	12	20	1
6	Control	22	39	0

Figure 30. Example of data series for repeated-event Cox analysis.

The length of the gray lines for each participant denotes the risk-time. As illustrated, a study participant could have no events (Subject 1, 4) but be censored at different times, either at the end of follow-up (Subject 1) or at the time of death or migration (Subject 4). A hospital admission can have different duration. Subject 2 thus does not contribute risk time during the time admitted, as indicated by the length of the black bar. In contrast, subject 5 is hospitalized slightly later and for a shorter time. After discharge from the hospital (if discharged alive), the subject enters the analysis again and goes back to contributing risk time (subjects 2, 3, 5, 6). To enable the analysis of the data in a recurrent-event model, a separate observation must be created in the database for the same infant, as illustrated in Figure 30 for the six infants depicted in Figure 29.

An infant that is never hospitalized therefore has 1 entry in the database, one that is hospitalized once has two entries, and subject 6 that is hospitalized three times would have four separate entries. Finally, subject 3 that was also hospitalized three times but died at the third hospitalization would have three separate observations in the analysis dataset.

4.2.3 Analysis of in-hospital case-fatality rates

In papers II and III, we evaluated the CFR (in-hospital mortality risk) among infants admitted to the hospital. Differences in CFR for admitted infants were calculated as cohort study risk-ratios using Stata's Tables for epidemiologists and tested for significance using Fisher's 2-sided exact test, which is an exact test that is useful for smaller samples of categorical data. Where the above Cox-analysis was used to test the risk of disease (morbidity) associated with different interventions, the approach described here was used to test whether an intervention was associated with changes in the *severity* of disease as expressed by the risk of in-hospital death among admitted infants.

4.2.4 Cox proportional hazards regression model

We assessed the all-cause mortality risk associated with different BCG strains (Paper III), BCG reaction characteristics (Paper IV), and TST conversions (paper V) using Cox survival models. The main assumption of the Cox-model is the assumption of proportional hazards, as indicated by the inclusion in the model's name. The important assumption is that the hazard risk in each group under evaluation is a constant multiple of the hazard risk in the other group(s) and that the hazard curves are thus proportional and do not cross. We tested the proportional hazards assumption by drawing log-log survival plots and by performing proportional hazards assumption tests based on Schoenfeld residuals (phtest). We furthermore conducted adjusted analyses by adding possible confounders one at a time and testing whether they modified the overall estimate of the intervention effect.

5 Results

Table 12. Overview of main results by thesis paper, adapted after A. Rieckmann. [174]

Paper	Exposure or comparison	Outcomes	Period	N	Events	Adjusted for (strata)	Statistical analysis	Association measure	Findings
I	Vaccine vials of BCG-Denmark and BCG-Russia	The number of neonatal doses that can be withdrawn from BCG-Denmark and BCG-Russia vaccine vials	Oct-Nov 2015	Vials: 39 BCG-Denmark 29 BCG-Russia	68 BCG vaccine vials opened	-	Linear regression	Correlation coefficient	Median doses obtained (should be 20 according to vaccine product insert): BCG-Denmark 13 (range 11-17) BCG-Russia 15 (range 12-17) More doses obtained from BCG-Russia than BCG-Denmark vials ($p < 0.00001$)
II	Three RCTs of BCG-Denmark versus no BCG to LBW neonates	Hospital admission risk In-hospital case-fatality	2002-2014	6,583 infants (3,297 in BCG group, 3,286 controls)	908 infant admissions, 135 in-hospital deaths	-	Multiple-event Cox proportional hazards Fisher's 2-sided exact test	BCG vs. control IRR, RR	Period: Admission IRR / Case-fatality RR Neonatal: 0.97 (0.72-1.31) / 0.58 (0.35-0.94) 6-week: 0.95 (0.73-1.24) / 0.56 (0.35-0.90) Infant: 0.96 (0.84-1.10) / 0.72 (0.53-0.99) Neonatal sepsis RR: 0.46 (0 .22–0.98)
III	RCT of vaccination at birth with BCG-Denmark vs. BCG-Russia (Phase I) and BCG-Japan vs. BCG-Russia (Phase II)	Hospital admission risk In-hospital case-fatality All-cause mortality risk BCG scar formation, TST responses & adverse events	2014-2018	I: 2,840 Denmark 2,837 Russia II: 3,184 Japan 3,160 Russia	I: 270 6-week admissions,77 deaths II: 346 6-week admissions, 60 deaths	-	Single and multiple-event Cox proportional hazard models Fisher's 2-sided exact test Binomial regression	Intervention versus BCG-Russia IRR, RR, MRR, PR, MD	The BCG strains did not affect admission risk or case-fatality. BCG-Japan tended to be associated with fewer deaths than BCG-Russia, the MRR being 0.71 (0.43-1.19). BCG-Japan and BCG-Denmark were both associated with more BCG reactions that were also larger, more BCG pustules and TST conversions, and more adverse events.
IV	Among BCG-vaccinated: Having a 2-month BCG reaction versus no reaction and reaction size	All-cause mortality and mortality due to infectious & noninfectious diseases between age 2-12-months	2002-2018	6,012 infants	119 deaths, 87 deaths due to infection.	Maternal MUAC, year, reaction assessor (RCT)	Cox proportional hazards	aMRR	Reactor/non-reactor aMRR 0.49 (0·26-0.95), linear association between ↑reaction size & ↓mortality. Effect strongest against fatal infection. Determinants for BCG reactions, size: Vac. technique, BCG strain
V	Among BCG-vaccinated: Having a TST reaction versus no reaction at 2-, 6 months of age	All-cause mortality between age 2-12-months and age 6-12-months	2008-2014	2 months: 2,105 infants 6 months: 4,618 infants	41 deaths between 2-12-months 13 deaths between 6-12-months	Age and place of vac., age at home-visit, mat. MUAC, assessor (RCT)	Cox Proportional Hazards	aMRR	TST versus no-TST aMRR up to 1 year of age Timing: Study / Meta-analysis 2 months: 0.34 (0.12-0.94) / 0.56 (0.38-0.83) 6 months: 0.68 (0.15-3.01) / 0.63 (0.39-1.00) TST determinants: Vac. technique, BCG strain

5.1 Paper I

The real-life number of neonatal doses of Bacille Calmette-Guérin vaccine in a 20-dose vial

In this small paper, we identified that commonly used BCG strains contain substantially fewer neonatal BCG doses than indicated on the vaccine package insert. We conducted the study because our vaccinators mentioned to us that it was never possible for them to withdraw 20 neonatal doses from BCG vials.

Over 29 days, the HNSM vaccinators counted the syringes used for vaccination separated by the vial strain (BCG-Denmark and BCG-Russia). After having completed the vaccination session, the vaccinators withdrew any remaining doses from the vials and counted the total amount of doses that had been withdrawn from each vial.

Rather than containing 20 neonatal doses of 0.05 ml, the vials of BCG-Denmark contained a median of 13 doses (range 11-17), while vials of BCG-Russia contained a median of 15 doses (range 12-17). More doses were obtained from the BCG-Russia vials than the BCG-Denmark vials ($p<0.00001$, Figure 31).

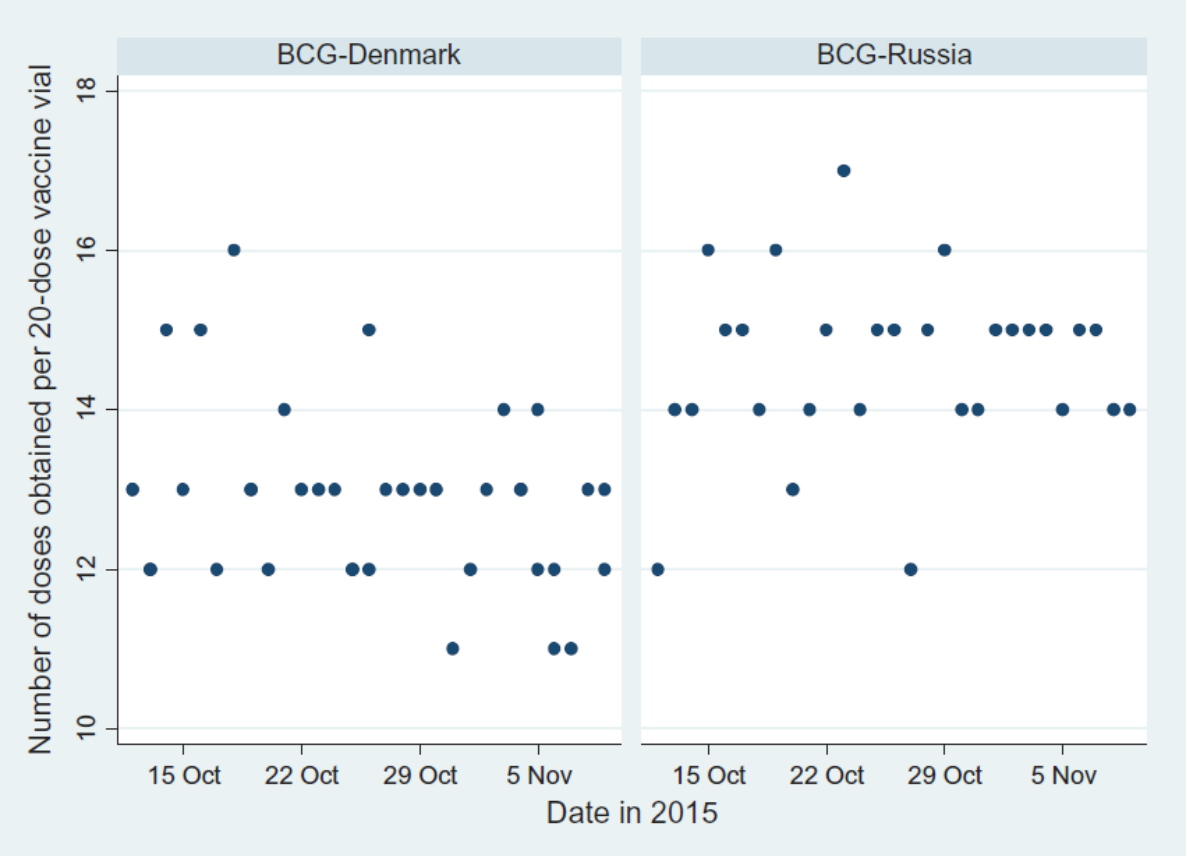


Figure 31. The number of doses obtained from 20-dose vials of BCG-Denmark and BCG-Russia.

There was no significant difference in the number of doses obtained by the two vaccinators, neither overall nor by vaccine type. The number of doses obtained per vial did not change over the study period, neither overall, by vaccine type, or by vaccinator.

5.2 Paper II

Early BCG Vaccination, Hospitalizations, and Hospital Deaths: Analysis of a Secondary Outcome in 3 Randomized Trials from Guinea-Bissau

The a priori hypothesis in the study protocol was that receiving early BCG would be associated with 50% fewer neonatal hospital admissions. Interestingly, there was no effect of BCG on the risk of admissions by neither 28 days, six weeks, or one year of age (Figure 32).

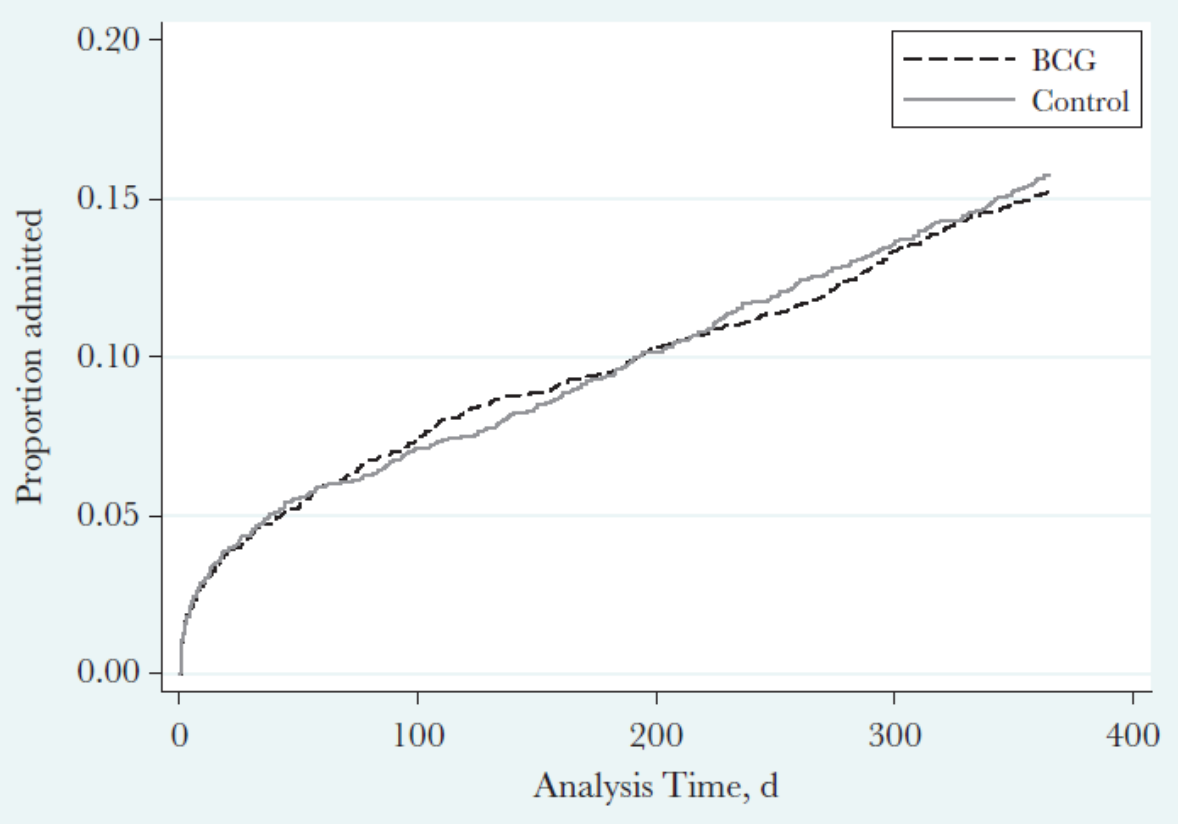


Figure 32. Kaplan-Meier cumulative hazards curve for hospitalization at Hospital Nacional Simão Mendes, combined for the 3 RCTs.

Instead, we found that BCG was associated with a tendency of fewer neonatal and infant admissions caused by sepsis, the IRRs being 0.75 (0.50-1.13) and 0.78 (0.55-1.11), respectively. Aside from this tendency, BCG reduced the all-cause case-fatality rate both in the neonatal and infant periods (Figure 33).

The effect on in-hospital case-fatality risk was particularly pronounced regarding the risk of fatal neonatal sepsis, from which our surveillance detected 7 BCG neonatal deaths vs. 20 control deaths.

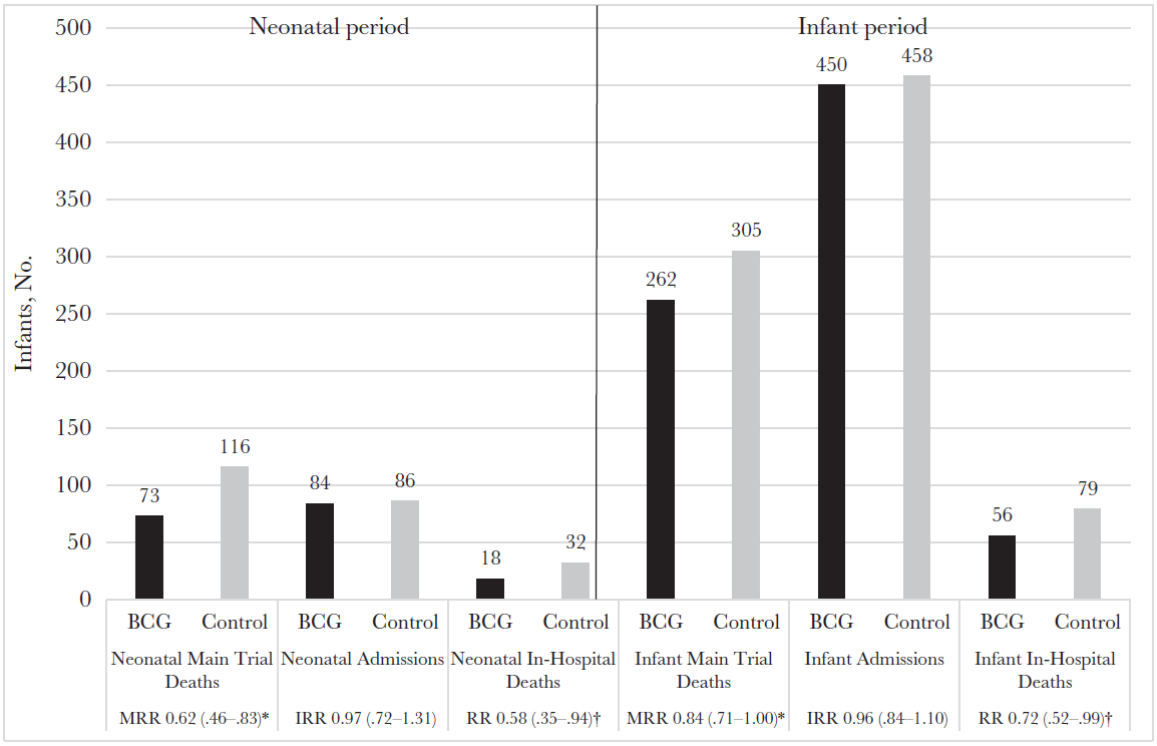


Figure 33. Mortality, hospitalization, and in-hospital mortality rates across the 3 RCTs of BCG versus no BCG. *P < 0.05 (main trial Cox analysis); †P < 0.05 (2-sided Fisher exact test).

Since there had also been slightly more admissions due to sepsis in the control group, BCG had reduced the neonatal in-hospital sepsis mortality rate by 54%, the RR being 0.46 (0.22-0.98). In contrast, the RRs for other infections and non-infectious diseases were not significantly different for BCG versus control infants (Figure 34).

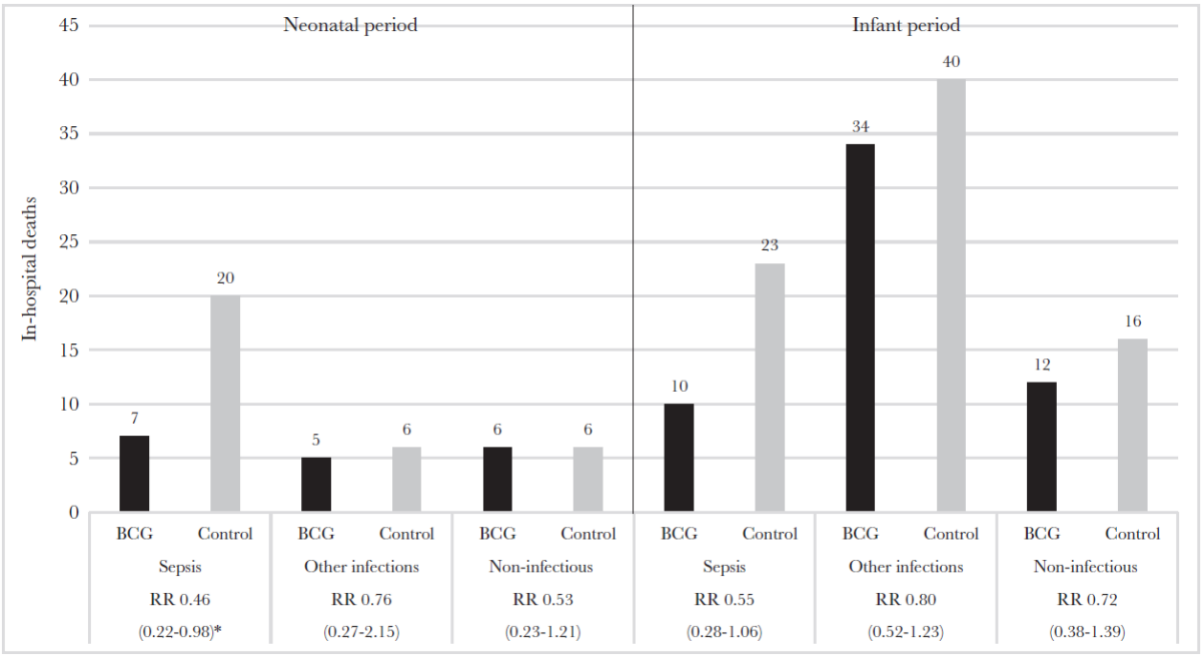


Figure 34. BCG versus control in-hospital deaths by disease and period of follow-up. *P < .05 (2-sided Fisher exact test).

Our surveillance registered one infant that was admitted under suspicion of TB. However, the infant was discharged with a final diagnosis of pneumonia, and the observed effects of BCG on in-hospital case-fatality risk are thus unlikely to have been caused by protection against TB.

5.3 Paper III

Early Vaccination With Bacille Calmette-Guérin-Denmark or BCG-Japan Versus BCG-Russia to Healthy Newborns in Guinea-Bissau: A Randomized Controlled Trial

The a priori hypothesis for this trial was that BCG-Denmark would be associated with 30% fewer hospital admissions by six weeks of age (before other vaccines with potentially immuno-modulatory effects are administered), when compared to BCG-Russia. This was based on the expectation that there would be an overall admission risk of 2.5%, and the sample size was thus 12,000 neonates (6,000 allocated to each BCG strain) corresponding to 258 events. As a secondary hypothesis, we expected that the overall mortality risk would be similar, e.g., 2.5%, and the study might thus have the power to show also a difference in mortality risk. Since the study was affected by the manufacturing halt of BCG-Denmark occurring in 2015, our last stock of BCG-Denmark expired by July 1, 2016, after 5,677 inclusions. We had applied the ethical committees and the DSMB for permission to exchange BCG-Denmark with BCG-Japan and complete the trial to the originally envisioned sample size of 12,000 as a natural experiment. In *Phase I*, which compared BCG-Denmark vs. BCG-Russia, there was a total of 270 hospital admissions by six weeks, e.g., a 4.8% (270/5,677) admission rate, almost twice as high as expected, while it was 5.5% (346/6,344) in Phase II. The corresponding BCG-Denmark versus BCG-Russia IRR was 1.08 (0.84-1.37) while the BCG-Japan vs. BCG-Russia IRR in Phase II of the trial was 1.15 (0.93-1.43). This effectively reproduces an important finding of Paper II; against our expectation, neither BCG nor BCG strains affect the admission risk to any degree in Guinea-Bissau (Figure 35).

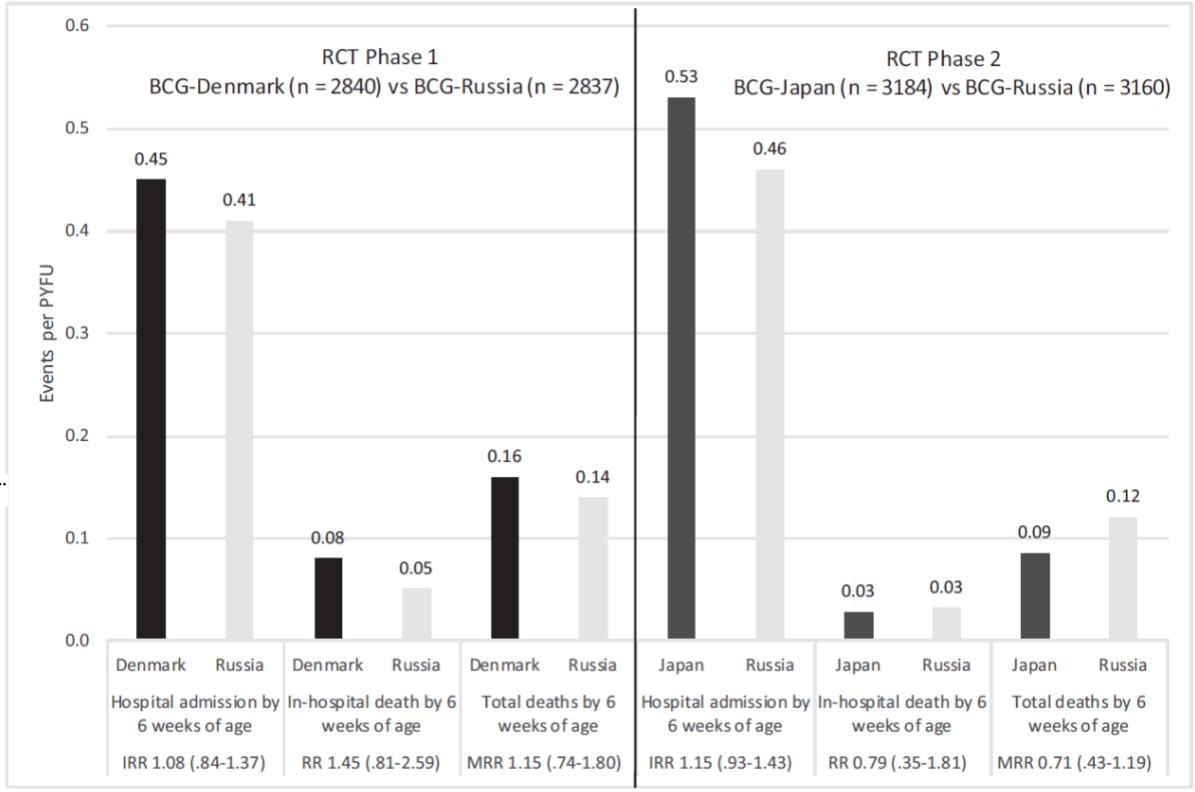


Figure 35. Hospital admissions, in-hospital mortality and total deaths across the two trial phases within the first six weeks of life.

Abbreviations: PYFU, Person-years of follow-up.

In terms of mortality risk, we estimated the risk of death by combining data collected by telephone, home-visit follow-up for the subgroup of infants from the HDSS, and data among infants admitted to HNSM. Both comparisons were underpowered for the mortality comparison due to the change in intervention and given that the mortality risk was lower than expected, being 1.4% (77/5,677) in Phase I and 0.9% (60/6,344) in Phase II. Nevertheless, there were some interesting clues in the data given that there was a tendency of slightly higher all-cause mortality risk and a tendency of higher in-hospital case-fatality for BCG-Denmark, while the opposite tendencies were seen for BCG-Japan (Figure 35).

Secondary outcomes were the BCG reaction prevalence, PPD (TST) responses and adverse events (left axillary lymphadenitis) among HDDS infants. The a priori hypotheses were an overall TST prevalence of 35% and power to show a 30% difference in TST and BCG scar prevalence related to one of the strains.

By two months of age, BCG-Denmark was associated with a higher BCG reaction prevalence, 99% (532/535) versus 96% (491/513) for BCG-Russia and BCG-Denmark skin reactions were on average 0.82 mm (0.67 mm to 0.96 mm) larger than BCG-Russia reactions (5.2 mm versus 4.3 mm). Likewise, BCG-Japan tended to be associated with more BCG reactions by two months, the prevalence being 98% (465/477) for BCG-Japan versus 96% (433/452) for BCG-Russia. BCG-Japan reactions were on average 0.71 mm (0.53 mm to 0.90 mm) larger than BCG-Russia reactions (5.4 mm vs. 4.7 mm). As expected, both BCG-Denmark and BCG-Japan tended to be associated with more adverse events, when compared to BCG-Russia.

By six months, the BCG reaction prevalence had waned substantially for infants that had received BCG-Russia, the phase I prevalence being 93% (382/412) for BCG-Russia vs. 99% (419/424) for BCG-Denmark, PR 1.07 (1.04-1.10), and the phase II prevalence being 92% (330/360) versus 97% (356/368) for BCG-Japan, PR 1.06 (1.02-1.09). The mean size of BCG reactions remained smaller for BCG-Russia in both comparisons. This indicates a long-lasting association between the BCG strain provided, reaction prevalence and reaction size, and that the smaller reactions associated with BCG-Russia are more likely to have disappeared by six months of age. For TST conversions, BCG-Denmark was associated with more TST reactions to both TST 2 TU and 10 TU, the BCG-Denmark/BCG-Russia PR being 3.19 (1.47-6.90) for 2 TU and 1.16 (1.02-1.32) for 10 TU. In Phase II, we exclusively used TST 2 TU, and BCG-Japan was associated with more TST conversions when compared to BCG-Russia, the PR being 1.84 (1.33-2.53) (Figure 36).

There were no cases of lymphadenitis associated with BCG-Russia.

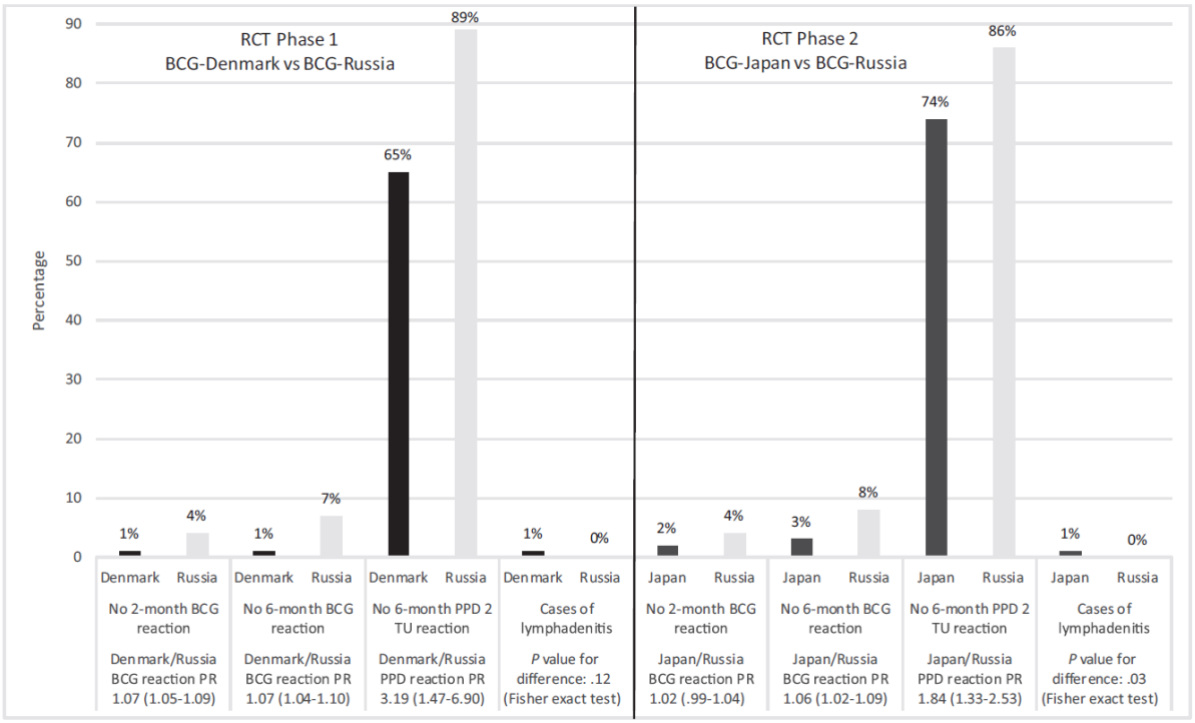


Figure 36. Percentage of HDSS infants that presented no BCG reaction, no TST reaction, and cases of lymphadenitis.

As an exploratory analysis, we evaluated the prevalence of BCG pustules by BCG strain with the hypothesis that more immunogenic BCG strains such as BCG-Denmark and BCG-Japan would be associated with more BCG pustules. We found that there were more BCG pustule skin lesions associated with BCG-Denmark and BCG-Japan, the BCG-Denmark/BCG-Russia pustule skin lesion RR being 1.26 (1.12-1.41) and the BCG-Japan/BCG-Russia RR being 1.15 (1.08-1.22). Pustules are larger than both papules and scars and might be associated with prolonged survival of BCG in the host and thus extended immune training, which we plan to pursue in a subsequent study when more early reaction data have been collected.

5.4 Paper IV

BCG skin reactions by 2 months of age are associated with better survival in infancy: A prospective observational study

In a large cohort of infants enrolled in five BCG RCTs conducted by BHP, we identified 8,103 infants that had received BCG within the first week of life. Of these, 6,012 were found at home at two months of age, where they had their early BCG skin reaction evaluated. The median age of BCG vaccination was one day, and our team had vaccinated 76% (4,551/6,012) of the infants within the first two days of life. The cohort was thus representative of the WHO recommendation of providing BCG shortly after birth. The 2-month BCG reaction prevalence was 97% (5,804/6,012) and 37% (2,213/6,012) of the infants had a small reaction (median diameter 3.5 mm), 28% (1,710/6,012) had a medium reaction (median diameter 5.0 mm) and 31% (1,881/6,012) had a large reaction (median diameter 6.0 mm).

The median 2-month MUAC was 122 mm among non-reactors and 124 mm for reactors (p<0.001). Among reactors, the median 2-month MUAC was 122 mm for small-reactors, 124 mm for medium reactors (p<0.001), and 128 mm for large-reactors (p<0.001). The BCG reaction rates were not influenced by ethnicity, maternal BCG scar status, birthweight group, or neonatal vitamin A supplementation. There were 119 deaths between 2 and 12 months of age in the cohort, and the mortality risk was 4.8% (10/208) for non-reactors and 1.9% (109/5,804), the aMRR thus being 0.49 (0.26-0.95) (Figure 37).

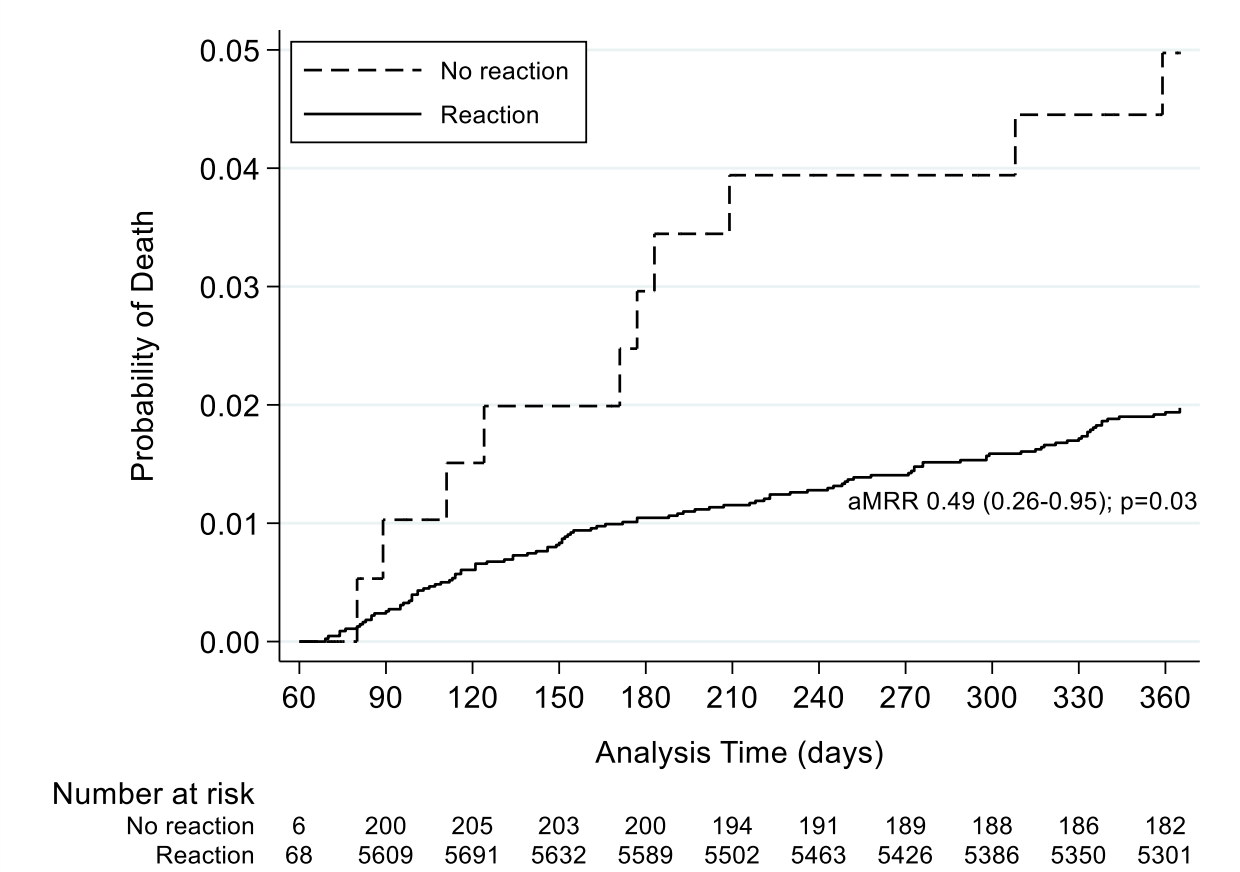


Figure 37. Kaplan-Meier Curve of Cumulative Deaths Up to 1 Year of Age Among Infants with a BCG Reaction Versus no Reaction.

By reaction size, the 2-12-month mortality risk was 2.9% (64/2,213) for small-reactors, 1.8% 23 (30/1,710) for medium-reactors and 0.8% (15/1,881) for large-reactors and the large/small 24 reactor aMRR was 0.35 (0.20-0.63) (Figure 38).

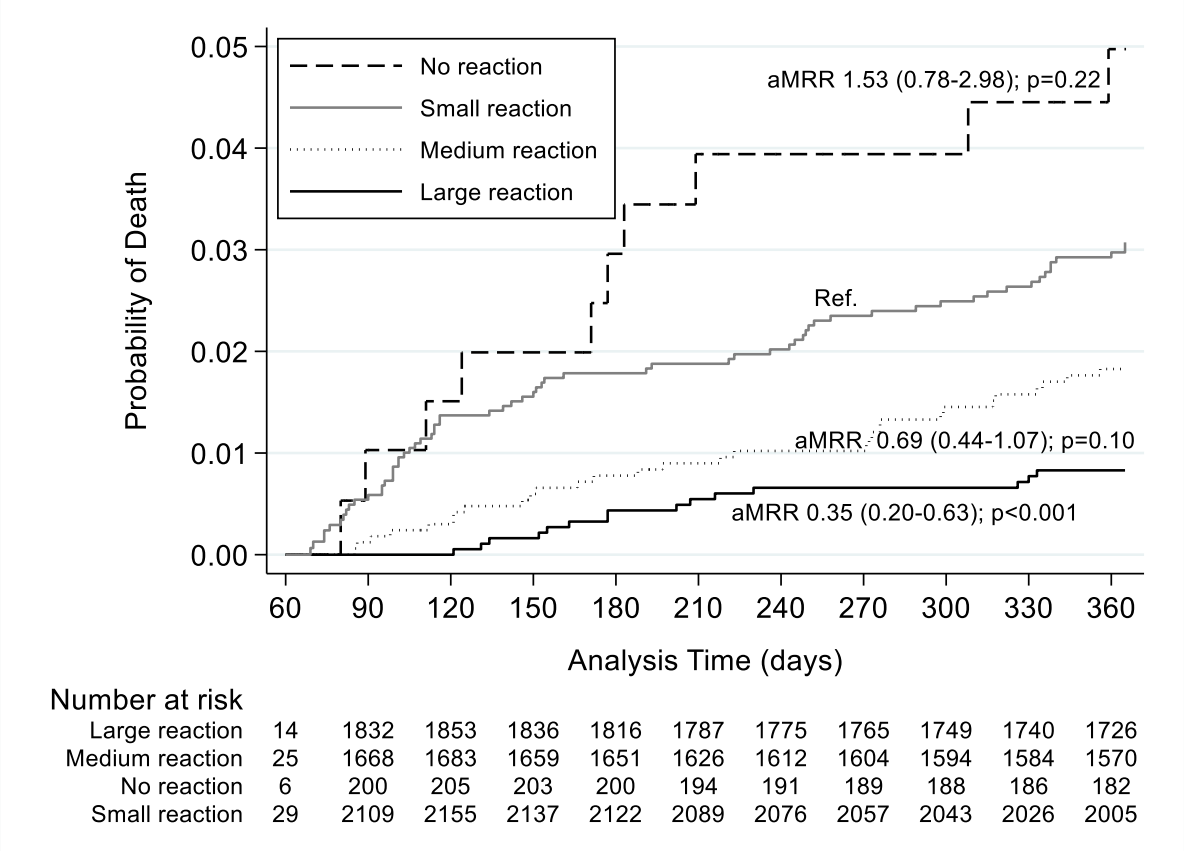


Figure 38. Kaplan-Meier Curve of Cumulative Deaths Up to 1 Year of Age by Reaction Size.

Interestingly, there was not only a reduced mortality associated with increased BCG reaction size for the tertiles we had divided them in; there was a linear correlation between increasing reaction size and decreasing all-cause mortality (Figure 39).

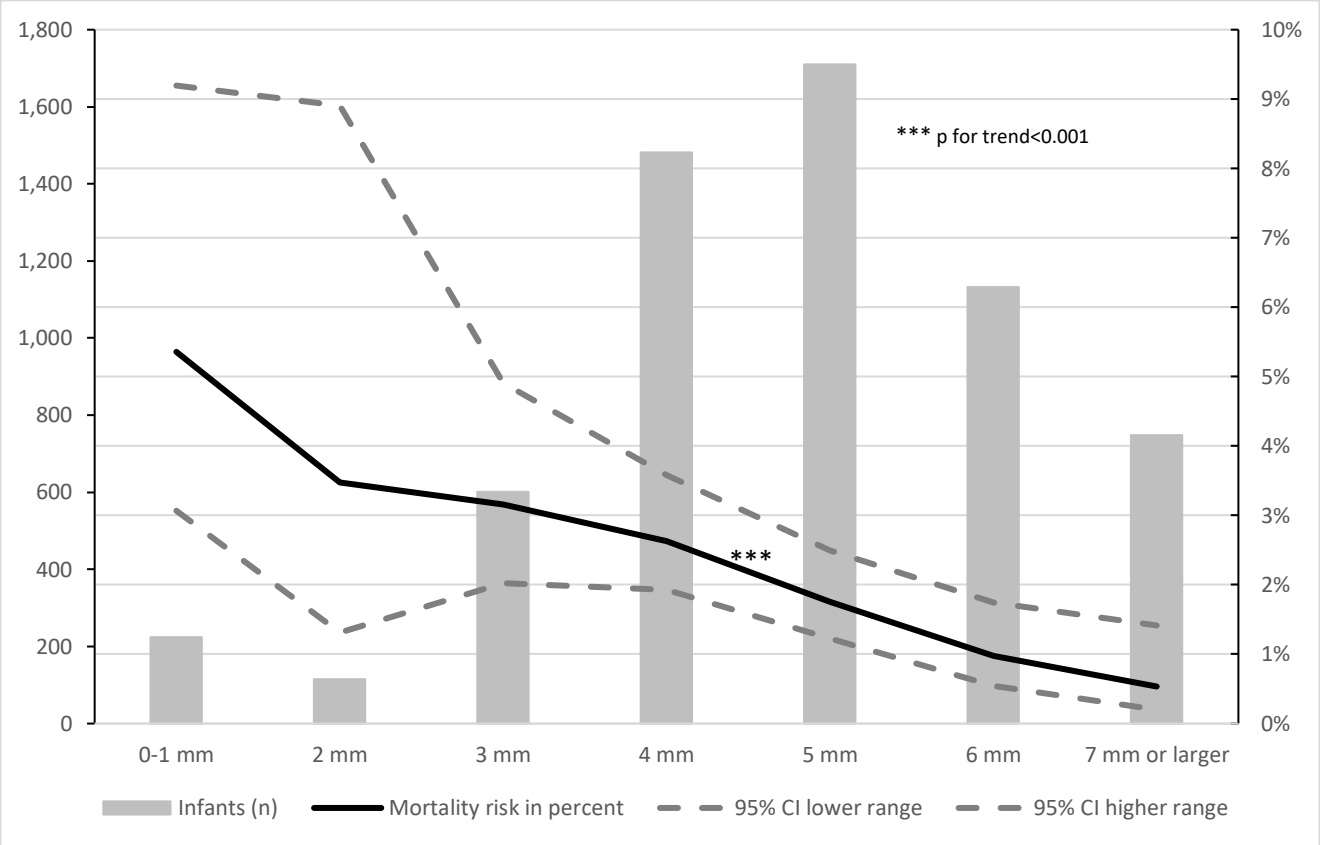


Figure 39. Mortality risk by reaction size in millimeters (CIs estimated by logistic regression).

These mortality effects were consistent across the RCTs, despite a trend of declining mortality occurring over the years that the different trials were conducted. All analyses were stratified by RCT and year.

We investigated causes of death in the cohort from verbal autopsies conducted three months after the child had died, from hospital admission data and information collected at the standardized house-visits. For the 119 deaths, 114 could be assigned a diagnosis; 23 could not be determined to be either infectious nor non-infectious since they were due to anemia (n=2), malnutrition/dehydration (n=11), sudden infant death syndrome (n=5) or they were undetermined (n=5). With a cautious approach that considers only deaths that could surely be attributed to be of infectious origin, 76% (87/114) of study deaths were caused by infection, and the reactor/non-reactor aMRR for infectious conditions was 0.53 (0.24-1.17).

Having a large versus small reaction was associated with a 68% reduced risk of death from infection, the aMRR being 0.32 (0.16-0.65), but no difference in the risk of death from non-infectious conditions, the aMRR being 0.65 (0.11-3.84) (Table 13).

Table 13. Infant cause-specific mortality rates and adjusted mortality rate ratios by 2-month BCG reaction status and BCG reaction size.

	Mortality Rate per 100 Pyrs (No. of Deaths/Total Pyrs)		Reactor/Non-reactor aMRR[a] (95% CI)	Mortality Rate Per 100 Pyrs (No. of Deaths/Total Pyrs)			aMRR[a] (95% CI)	
	No Reaction	Has Reaction		Small Reaction	Medium Reaction	Large Reaction	Large vs Small	Large vs Medium
Infectious Conditions	4.4 (7/159)	1.8 (80/4504)	0.53 (0.24-1.17)	2.9 (49/1704)	1.6 (21/1333)	0.7 (10/1466)	0.32 (0.16-0.65)	0.48 (0.23-1.03)
Non-infectious Conditions[b]	0.0 (0/159)	0.2 (9/4504)	NA	0.2 (4/1704)	0.2 (3/1333)	0.1 (2/1466)	0.65 (0.11-3.84)	0.68 (0.11-4.22)

Within the LBW RCTs, neonates were recruited at HNSM and HDSS health centers. The vaccinators at the health centers would not apply BCG every day like at HNSM, where the vaccinators have provided BCG to approximately 20 neonates per day for years. Accordingly, the risk of developing no BCG reaction was higher for infants included at the smaller health centers than for those included at HNSM, the Health Center/HNSM RR for no reaction being 2.43 (1.50-3.92).

For a portion of the cohort, it was registered which HNSM vaccinator had applied BCG. There were differences between them: the mean size of the post-vaccination wheal was 4.3 mm for vaccinator 1 and 4.5 mm for vaccinator 2 ($p<0.001$). The risk of developing no reaction was 4% (49/1,263) for vaccinator 1 versus 1% (6/695) for vaccinator 2, the vaccinator 1/vaccinator 2 RR for no reaction being 4.49 (1.93-10.4). Finally, smaller post-vaccination wheal sizes were associated with a higher risk of developing no subsequent BCG reaction. The most important determinant for developing a BCG reaction and the reaction size was the BCG strain provided, however (Table 14).

Table 14. Influence of selected determinants on the prevalence of 2-month BCG reactions and the BCG reaction size at two months of age.

Determinant	Specification	No Reaction RR (95% CI)	Small Reaction	Small Reaction RR (95% CI)	Medium Reaction	Medium Reaction RR (95% CI)	Large Reaction	Large Reaction RR (95% CI)
Post-vaccination wheal size	Small	5.91 (2.62-13.3)	32% (72/227)	1.07 (0.86-1.33)	26% (58/227)	0.79 (0.62-1.00)	36% (82/227)	0.98 (0.81-1.19)
	Medium	3.01 (1.44-6.29)	35% (318/921)	1.17 (1.02-1.34)	29% (271/921)	0.90 (0.79-1.04)	33% (301/921)	0.89 (0.78-1.01)
	Large	Ref.	30% (238/805)	Ref.	33% (262/805)	Ref.	37% (296/805)	Ref.
Vaccinator	1	4.49 (1.93-10.4)	32% (405/1,263)	0.98 (0.86-1.12)	30% (379/1,263)	0.98 (0.85-1.13)	34% (430/1,263	0.95 (0.84-1.08)
	2	Ref.	33% (228/695)	Ref.	31% (213/695)	Ref.	36% (248/695)	Ref.
BCG strain	Denmark	Ref.	24% (126/534)	Ref.	31% (168/534)	Ref.	44% (237/534)	Ref.
	Russia	7.68 (2.31-25.5)	47% (240/510)	1.99 (1.67-2.38)	32% (163/510)	1.02 (0.85-1.21)	17% (85/510)	0.38 (0.30-0.47)
BCG strain	Japan	Ref.	22% (103/470)	Ref.	27% (128/470)	Ref.	48% (227/470)	Ref.
	Russia	1.57 (0.77-3.23)	37% (164/448)	1.67 (1.35-2.06)	30% (134/448)	1.10 (0.89-1.35)	29% (132/448)	0.61 (0.51-0.72)

A subgroup of infants that were included in the first large LBW RCT was also visited at four weeks of age. Given that informed consent was provided, our team collected blood samples at four weeks of age for immunological assessment, and the infant's developing BCG reaction was examined.[175] For the 224 infants that had been bled and where BCG reaction data were available, we examined whether the presence and size of BCG reactions influenced immune responses.

Interestingly, the 4-week BCG reaction prevalence was 92% (205/224) and having a 4-week BCG reaction was associated with a higher monocyte cell count, 1,760/µL [1,340-2,330] for reactors versus 1,240/µL [950-1,830] for non-reactors (p=0.02) and the neutrophil cell count tended to be higher as it was 2,070/µL [1,450-3,010] for reactors versus 1,850/µL [790-2,610] for non-reactors (p=0.19). BCG reactors had higher responses to stimulation with PPD, especially IFN-γ (GMR=4.62 [1.70-12.4]), when compared to non-reactors. A comparable pattern was seen after stimulation with heterologous TLR agonists, whereas both unstimulated and PMA stimulated responses were generally higher among non-reactors (PMA: IFN-γ: GMR=0.80 [0.75-0.87]; unstimulated: IL-1β: GMR=0.40 [0.17-0.95], IL-6: GMR=0.33 [0.14-0.79], TNF-α: GMR=0.51 [0.30-0.87], IL-17: GMR=0.44 [0.20-0.95] and IL-10: GMR=0.45 [0.21-0.96]). Among all stimulus cytokine combinations, a larger reaction was generally associated with higher cytokine responses.

5.5 Paper V

Neonatal Bacille Calmette-Guérin Vaccination and Tuberculin Skin Test Reactions at 2- and 6-Months: Effects on Mortality up to 1 Year of Age

By two months of age, TST data was available from the small LBW RCT conducted between 2002-04 and the most recent large LBW trial conducted between 2008-2014.[8,141]

The prevalence of positive TSTs among infants that had received BCG in the neonatal period within the two RCTs was 22% (302/1,389). The 2-12-month mortality risk was 1.7% (5/302) for reactors and 3.3% (36/1,087) for non-reactors, the corresponding crude reactor/non-reactor MRR being 0.49 (0.19-1.26) and the aMRR being 0.34 (0.12-0.94) (Figure 40A).

By six months of age, TST data was available from the two studies mentioned above plus the BCGSTRAIN I trial.[67] The prevalence of positive TSTs at six months was 44% (1,149/2,635). The 6-12-month mortality risk was 0.4% (4/1149) for reactors and 0.6% (9/1486) for non-reactors, the corresponding crude reactor/non-reactor MRR being 0.87 (0.27-2.86) and the aMRR being 0.68 (0.15-3.01) (Figure 40B).

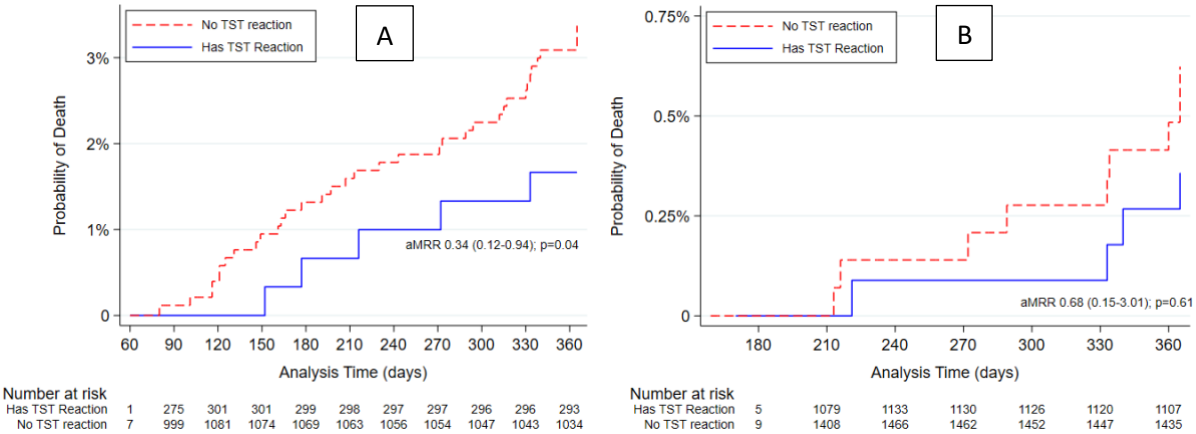


Figure 40 A+B. Kaplan-Meier curves of cumulative deaths up to 1 year of age among infants with a 2-month (panel A) or 6-month (panel B) TST reaction versus no reaction.

We conducted a literature search to identify studies that have evaluated the association between TST reaction status and subsequent all-cause mortality among BCG-vaccinated infants, using prospective follow-up. We identified three observational studies that were all from Guinea-Bissau and comprised four cohorts of BCG-vaccinated infants that had TST assessments performed at 2- and 6-months of age.[5–7]

Across the studies, there was data on a total of more than 10,000 TST evaluations, of which 28% (1,494/5,409) were positive at two months of age, and 42% (2278/5419) were positive at six months of age (Table 15).

The combined estimate (fixed effects) for having a 2-month TST reaction versus no reaction was an aMRR of 0.56 (0.38-0.83), while the 6-month estimate was 0.63 (0.39-1.00) (p for same effect=0.71) (Figure 41).

Table 15. Studies reporting prospective mortality by 2- or 6-month TST reaction status.

Study	Infants TST Evaluated (n)	TST Dose Applied	Prevalence of TST Reactions	Period of Follow-up	Reactor/nonreactor aMRR (95% CI)
TST reactions by two months of age					
Epidemiology 2006[6]*	1566	2 TU	30% (465/1566)	2-18 months	0.54 (0.29-0.99)
TMIH 2015 LBW[7]	803	2 TU	16% (132/803)	2-12 months	0.47 (0.14-1.54)
TMIH 2015 NBW[7]	1651	2 TU	36% (595/1651)	2-12 months	0.75 (0.40-1.42)
Present study	1389	2 TU	22% (302/1389)	2-12 months	0.34 (0.12-0.94)
Combined estimate (fixed effects)	5409	2 TU	28% (1494/5409)	-	0.56 (0.38-0.83)
TST reactions by six months of age					
Vaccine 2003[5]	813	Undeclared	59% (479/813)	7.5-19.5 months	0.48 (0.25-0.90)
TMIH 2015 LBW[7]	619	2 TU	27% (171/619)	6-12 months	0.52 (0.12-2.33)
TMIH 2015 NBW[7]	1,340	2 TU	35% (474/1,340)	6-12 months	1.13 (0.45-2.86)
Present study	2,635	2 and 10 TU	44% (1,149/2,635)	6-12 months	0.68 (0.15-3.01)
Combined estimate (fixed effects)	5,419	2 and 10 TU	42% (2,278/5,419)	-	0.63 (0.39-1.00)

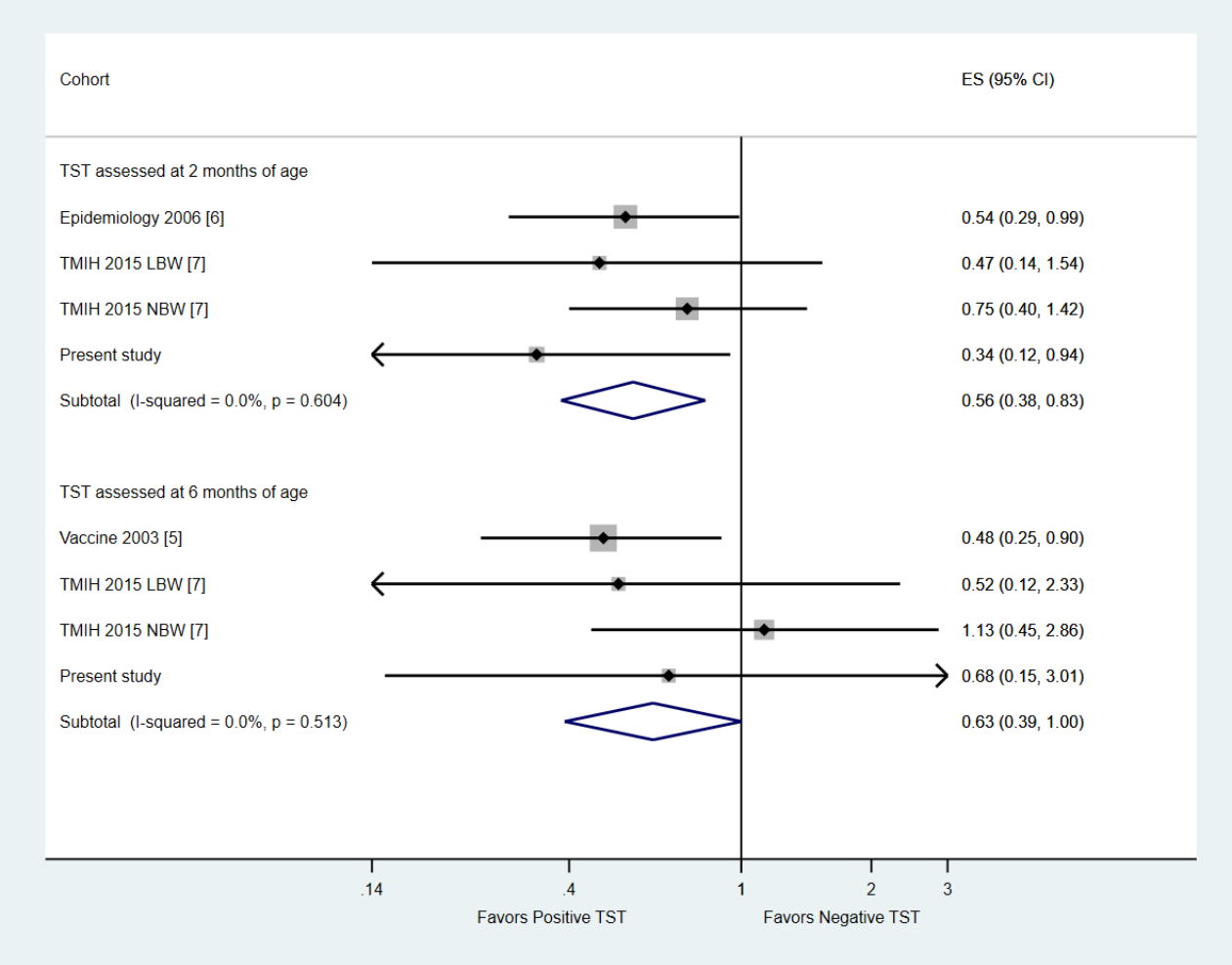


Figure 41. Forest plot of studies evaluating the association between having a TST reaction versus no reaction at two and six months of age.

For TST reactions by two months of age, none of the tested determinants were associated with subsequent TST reactions, but the weight at inclusion tended to be associated with developing a reaction (p=0.06). For TSTs at six months of age, Maternal MUAC, age, and BCG scar status were not associated with reactions, and neither was inclusion weight. Vaccinator 1 tended to be associated with more TST conversions and the age at BCG vaccination was associated with TSTs, the prevalence of reactions for infants vaccinated $\leq$3 days after birth being 36% (577/1,601). In comparison, it was 31% (132/427) for infants vaccinated later in the neonatal period, the positive TST reaction RR for early versus late BCG being 1.17 (1.00-1.36). The post-vaccination wheal size plus the BCG strain were strongly associated with higher TST reaction prevalence by six months, BCG-Denmark, and BCG-Japan producing substantially more TST reactions when compared to BCG-Russia.

6 Discussion

The available historical observations regarding BCG, the more recent observational studies and RCTs all indicate that BCG has beneficial NSEs.

The present thesis corroborated and extended these findings by showing that BCG does not affect the risk of hospitalization, but had marked effects on the mortality risk among hospitalized infants and on the risk of fatal neonatal sepsis. The present thesis also demonstrates that BCG reaction characteristics and TST reactions positively affect the subsequent all-cause mortality risk. Randomized data from a large-scale trial demonstrated that the main determinant behind these beneficial reactions were the BCG strain administered.

The data presented in papers III-V and recent work presented by other groups[48,88], most notably related to the number of viable mycobacteria in different BCG preparations, thus indicate that BCG strains likely have differential NSEs.

The present data do not allow for any definitive conclusions but provide a roadmap for additional RCTs and laboratory studies. Separate questions regarding the specific protection provided against TB, adverse events, and a possible shortage of specific BCG strain(s) would have to be addressed to reach the end goal of having a BCG vaccine strain in adequate supply to reach world demands that has optimal TB-specific and non-specific effects.

6.1 Strengths and weaknesses

6.1.1 Paper I

The real life number of neonatal doses of Bacille Calmette Guerin vaccine in a 20 dose vial

In this study, we evaluated only vials BCG-Denmark and BCG-Russia for the dose content that can be extracted from the vials. The two vaccine strains are delivered with the lyophilized vaccine preparation in one vial and approx. 1 ml of diluent in a separate vial, which is to be transferred to the vaccine vial to reconstitute the vaccine. The same principle applies to BCG-Japan, BCG-Bulgaria, and BCG-GreenSignal.

A major limitation of our study is that two very trained vaccinators handled the vials. The two had vaccinated with BCG since before 2002 and worked with the BHP at HNSM from 2002. With 6,000-7,000 births per year and a similar amount of BCG vaccinations divided between them, there is every reason to believe that they are both very skilled at handling the vaccine. This is demonstrated by the very high BCG reaction prevalence in the cohorts they vaccinated – these cohorts feature the highest BCG reaction prevalences published in the literature. It is thus likely that our results reflect close to the maximum number of doses that can be withdrawn, which is important information. But it also might not reflect how much more average-skilled vaccinators can withdraw. We might thus have effectively provided an overestimation of the number of doses.

The difference in doses that can be withdrawn from the two vials was robust over time and by the vaccinator. This difference could thus likely be caused by a slightly higher volume of diluent shipped with BCG-Russia, or perhaps either the diluent or the vaccine vial is shaped in a way that facilitates the withdrawal of slightly more liquid than for BCG-Denmark. A way to have investigated these questions further and brought forward additional knowledge about the different steps of the vaccine reconstitution process would have been to weigh the different parts (syringes, vials) on a precision weight. With such an approach, we could have estimated the liquid wastage in the dead spaces of the diluent vial, the syringe used to transfer the diluent, the vaccine vial, and the syringes used for injection. If vaccine wastage could be reduced at any of these steps, then BCG doses would be freed for their real purpose.

BCG-Denmark (**Figure 42**A), BCG-GreenSignal (**Figure 42**B), and BCG-Russia (**Figure 42**C) are all delivered as enclosed vaccine vials with a soft enclosed cap through which the diluent is transferred with a syringe. In addition, the diluent vial for BCG-Denmark is also capped. For BCG-GreenSignal and BCG-Russia, the diluent vial is a medication-like bottle to be snipped, while both the diluent and vaccine vial for BCG-Japan (**Figure 42**D) and BCG-Bulgaria (**Figure 42**E) is to be snipped, "*open bottles*".

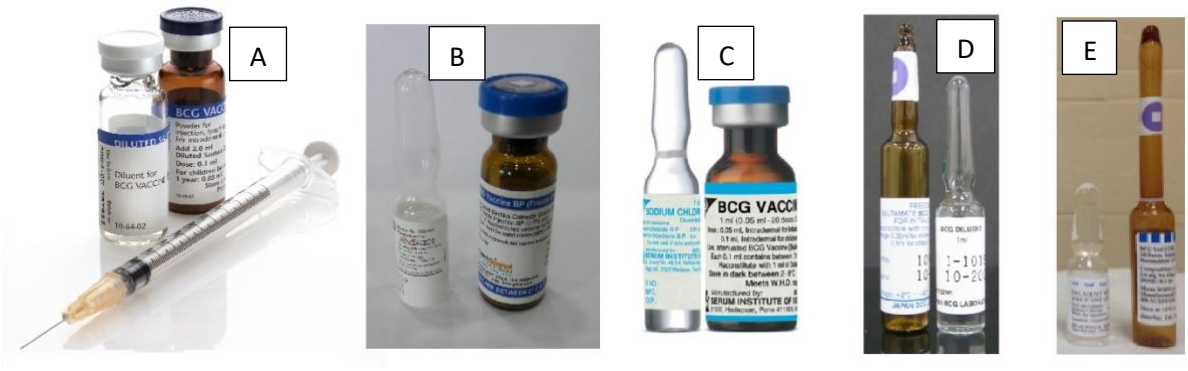


Figure 42 A-E. WHO prequalified BCG vial preparations.

From personal experience, some liquid remains in the area close to the cap for BCG-Denmark (Figure 42A) – both in the diluent bottle and in the vaccine bottle - which could perhaps explain our finding of slightly fewer doses in the BCG-Denmark preparation, when compared to BCG-Russia. We did not test whether BCG-Russia is shipped with slightly more

diluent volume, however. While it might be easier to withdraw liquid from the open bottles, it might also be easier to spill doses as well, especially for less experienced vaccinators. Certainly, the last couple of doses are retracted while the vial is held almost upside-down. This is challenging and might result in spills in the hands of less-experienced personnel. Since we did not test vaccine preparations delivered as "open bottles," it is unknown whether these preparations are associated with similar actual vial dose contents. From personal communication with the manufacturers of BCG-Japan, it is however clear that BCG-Japan does not contain 20 doses in real-life either.

It is impossible to withdraw and administer 20 doses of exactly 0.05 ml when having exactly 1 ml of liquid provided to begin with. Eliminating wastage in the dead space of the diluent and vaccine vials and syringes used is not feasible. Testing whether different vial- and syringe types are associated with differences in wastage might be an idea to reduce wastage of BCG.

BCG production is a complicated process with relatively low economic attractiveness, which has meant that the vaccine supply has been unstable, especially after the production of BCG-Denmark ceased.[37] In 2017, the available supply of BCG vaccine was forecasted to be 500m doses, while the forecasted demand was 350m doses.[37] This is in sharp contrast to the actual number of humans vaccinated with BCG per year, reported to be around 120m.[13] A part of this discrepancy will be due to unused doses in the vials and unused (expired) vials. Still, a substantial source of this "wastage" is likely caused by vials containing only 13-15 doses per vial, rather than 20 doses.

6.1.2 Paper II

Early BCG Vaccination, Hospitalizations, and Hospital Deaths: Analysis of a Secondary Outcome in 3 Randomized Trials from Guinea-Bissau

Strengths of this study include the randomized design and the large sample size of 6,583 infants, which meant that there were 908 infant hospitalizations and 135 in-hospital deaths to be included in the analysis. The main BHP data entry supervisor at the HNSM Pediatric Ward is dedicated to the job and has worked with BHP since the 1980s, maintaining the database used for the study since January 1, 2001, and the data have been used for a series of BHP studies.

One strength is that the diagnoses used for the analysis of cause of death at the hospital were given by the physicians that had been involved in the treatment of the infants, as opposed to verbal autopsies where a physician retrospectively analyzes information collected in a standardized manner, and assigns a probable diagnosis. However, the limited diagnostic tools available at the HNSM Pediatric ward is a limitation in the precision of these diagnoses. When limited diagnostic tools are available, doctors must rely on their skills as clinicians. We focused on the risk of sepsis and other infections, and the signs and symptoms of such infections and their high frequency at the ward and fairly clear symptomatology suggest that they would have been recognizable. Most doctors in Guinea-Bissau have been trained at the university in Guinea-Bissau, which is run by Cuban doctors that have a recognition for being skilled clinicians.

The major limitation of the study is that we might have had incomplete registration of infants. At the time of data collection, the data registration was largely based on the journal charts used by the doctors for the treatment of the patients. Infants that were hospitalized for a short time or that died shortly after admission might not have had a chart made for them. Separate issues relate to infants admitted during the night and discharged (dead or alive) in the early morning. Also, not all infants would have an exit status registered on their chart, or the chart might have disappeared during the admission, which means that we had incomplete data in terms of whether the infant survived the admission or not. We attempted to ensure that all exit statuses were registered correctly by analyzing verbal autopsies. This was done for infants registered to have died within one week after hospital discharge and we identified several additional infants that had died at the hospital in this manner. If the infant was lost to follow-up in the main trial, this would, however, not be possible. Also, we did not include infants that had been hospitalized after loss to follow-up in the main trial, since a differential loss to follow-up in the intervention and control groups could have then affected results. We thus excluded infants in the hospital admission analysis at the time that they had moved or migrated, despite that they could have still been hospitalized and died at HNSM, but this would probably not constitute a substantial issue for neonatal outcomes.

A study is only truly randomized right after randomization. In this study, several factors could have reduced the difference between the groups. First, cross-over from the control group to the BCG group due to mothers procuring BCG for their child will have reduced any differences between the groups. This was probably not a significant factor, however, since just 3% (25/895) had received BCG by 14 days of age and 11% (104/916) by four weeks of age in the 2004-08 RCT.[140]

Since the mortality was higher in the control group than in the BCG group, BCG has likely saved the frailer infants that would have otherwise died, had they not received BCG. Such frail infants might have accumulated more risk-time in the BCG group than in the control group, and they would likely have had a higher risk of hospital admission, which could have reduced the BCG versus control difference in admission risk. Hence, death was a competing risk that might have affected outcomes, but the effect was probably limited.

When infants arrive at the Pediatric ward, the mother or caretaker brings the vaccination card of the child in approximately 60% of cases. When the vaccination card is available, our assistants register all available information, including unique BHP RCT ID number, area of residence, HDSS ID (if applicable), parental names, the infant's name, birthdate, and vaccination dates. Data linkage is thus straightforward. For the 40% where information from the vaccination card is not registered, we used a series of manual data cleaning procedures to identify additional admissions. Parents sometimes cannot remember the birthdate of the child, and it is also sometimes the uncle or aunt that brings the child to the hospital. There have therefore inevitably been situations where it was not possible to confirm without reasonable doubt that the infant was, in fact, a study infant. In such cases, the child was not included in the analysis.

On a similar note, our team was present to register information for infants admitted between 08-20 each day in the triage room. Since hospital admissions occurring at odd hours are likely more severe than those occurring during day-time, severe admissions might have been less likely to be captured. Some periods have likely been affected by a power outage occurring intermittenly, but we did not test whether there were periods in the data with no study hospital admissions. Also, while the pediatric ward at HNSM is the largest in the country, infants can have been admitted to other smaller health centers from which we had no data on hospital admissions.

The was thus missing records at HNSM and a lack of bed-side confirmation of events (which was introduced later) and overall limitations in the data collection for our analysis. These factors would likely lead to an underestimation of the effects of early BCG versus no BCG. Likewise, all infants received OPV on the day of inclusion, and a series of studies have indicated that OPV has beneficial NSEs, including an RCT of providing OPV at birth versus no-OPV.[156] If OPV has beneficial NSEs, then OPV could have reduced the measurable difference between intervention and control, especially if OPV induces its beneficial effects on the immune system via some of the same pathways as BCG. In the RCTs providing BCG-Russia conducted in India, the first trial was without coadministration of OPV and reported a BCG/control estimate of 5% (-13% to 20%), while the second trial of BCG-Russia+OPV versus control alone reported an estimate of -1% (-23% to 17%) (p for same effect=0.65). As such, it is unclear whether co-administration of OPV might have reduced the beneficial effects of BCG; in an RCT from Bissau, receiving BCG + OPV versus BCG only was associated with a 42% reduction in mortality for infants enrolled with two days of birth, the MRR being 0.58 (0.38-0.90).[156]

Finally, given the recent developments regarding the appearance of a mechanism behind BCG's NSEs in terms of EG, it would have been useful to analyze admission patterns and in-hospital deaths by three days of age, rather than only by four weeks, six weeks and one year of age.

6.1.3 Paper III

Early Vaccination With Bacille Calmette-Guérin-Denmark or BCG-Japan Versus BCG-Russia to Healthy Newborns in Guinea-Bissau: A Randomized Controlled Trial

Many of the strengths and weaknesses discussed for paper II apply to paper III as well, given the similarity in design and the fact that hospital admissions at HNSM was the primary outcome of the trial.

There are also some important differences, however:

First, we implemented bed-side confirmation of events at the Pediatric Ward at the beginning of 2016 (approx. when two-thirds of the Phase I BCG-Denmark versus BCG-Russia comparison had been completed). This meant that daily rounds were done twice per day to register new admissions and that bed-side confirmation of vital status was performed. If an infant had been discharged and left the facility, then our assistant would consult with neighboring beds whether the infant was discharged alive or had died during the admission.

In case that the information could not be obtained, the data entry supervisor would telephone the family to ask whether the infant was discharged alive or not.

Second, Doctors Without Borders (MSF) initiated a 4-year intervention at the Pediatric ward from April 2016, which mainly included that Spanish medical personnel were present at the Ward and also involved education and capacity building with hiring and training of local nurses and doctors, reconstruction of the facility and changes in the triage procedures. A separate Neonatal Intensive Care Unit was constructed with approximately 40 beds and kangaroo mother care was introduced. The intervention meant that more infants were admitted in early life (before six weeks of age) while fewer were admitted from six weeks to one year of age, and the in-hospital case-fatality rate fell substantially, from approx. 15% to 5%. The combined effects of these interventions were detectable in our data, in which the hospital admission frequency for the two phases were 4.8% (270/5,677) and 5.5% (346/6,344) and the case-fatality risk 15% (41/270) in phase I and 6% (21/346) in phase II of the RCT.

The higher early-life admission frequency following the MSF intervention increased our power slightly, but it could also mean that more neonates were hospitalized as a precaution due to their age rather than due to signs of sickness. The substantial reduction in the in-hospital case-fatality rate made it more difficult to show a difference between BCG strains. It might have also lowered the overall trial mortality, reducing the statistical power.

Aside from the hospital admission data, we used a combination of telephone follow-up (to all infants with a telephone number registered) and home-visits to infants residing in the HDSS to access secondary outcomes. We conducted a combined analysis of the effects of the different BCG strains on all-cause mortality by combining the mortality data collected by telephone, within the HDSS and at HNSM for infants with neither telephone nor HDSS follow-up. This approach and the fact that some mothers did not provide any telephone numbers at inclusion or only one number, which carried a high risk of unsuccessful follow-up, meant that 2,025 infants (17% of the cohort) were not reached by home-visits or telephone follow-up. Among these, there were 73 six-week admissions and eight deaths at the hospital. There was not information available for infants that died outside of the hospital that were lost to follow-up.

A major strength of the study is the experience in conducting RCTs and collecting data that all members of the team possessed. We were blessed with skilled assistants and supervisors that have worked for BHP for many years. The capacity to conduct large-scale RCTs has been built over many years by many researchers, in close collaboration with assistants.

The change in BCG strain might be regarded both as a blessing and a curse. It meant that the study became even more restrained, especially for the mortality comparison, while at the same time contributing important data regarding BCG-Japan. Due to the higher-than-expected hospital admission frequency, we did have the power to evaluate the effects by strain on this outcome, and there were no significant differences between the strains.

Based on the existing literature, when the study protocol was written, we expected much less BCG scars associated with BCG-Russia than there actually was. While our study did show

a reduced prevalence of BCG scars associated with BCG-Russia, it also demonstrated that it is possible to obtain early BCG reaction rates of >97% even for BCG-Russia when using adequate vaccination technique. The high vaccination quality may indicate that even a relatively weaker strain such as BCG-Russia was able to induce a considerable effect. The reaction prevalence was thus high, even when compared to what has previously been reported for more immunogenic strains.[20,21] Given the substantially lower content of viable mycobacteria in BCG-Russia, one might speculate that the delivery of, say, 20-30% less BCG liquid due to substandard vaccination procedures may have more consequences when using BCG-Russia than BCG-Japan. This could be because even the delivery of a reduced dose would likely be effective if administering BCG-Japan, whereas it would be ineffective with BCG-Russia. The quality of BCG delivery could potentially have concealed the differences between the strains, which might have been more pronounced in the hands of more averagely skilled vaccinators.

Important aspects of BCG reaction kinetics, in particular for BCG-Russia, which is associated with smaller BCG reactions, fewer BCG pustules, and *waning* of BCG reactions (Table 16), had not been included in the study protocol. We were unaware of these effects and it is likely that they could be discovered because of the high quality of the vaccinations provided and the experience of our follow-up assistants.

Table 16. Prevalence of BCG reactions by 2- and 6-months of age, by BCG strain and study phase.

	2-month prevalence	6-month prevalence	Reaction waning in %
BCG-Denmark	99% (532/535)	99% (419/424)	0%
BCG-Russia, Phase I	96% (491/513)	93% (382/412)	-3%
BCG-Russia, Phase II	96% (433/452)	92% (330/360)	-4%
BCG-Japan	98% (465/477)	97% (356/368)	-1%

6.1.4 Paper IV

BCG skin reactions by 2 months of age are associated with better survival in infancy: A prospective observational study

Principal strengths of this study are the large cohort of 6,012 infants that had all received BCG very shortly after birth, allowing time for the BCG reactions to have developed by two months of age. This also made it possible to compare characteristics of these reactions, including the sizes and analyze determinants of reaction kinetics without the age of vaccination as a potential confounder. Furthermore, the official recommendation is to provide BCG at birth in countries where TB and/or lepra is endemic.[1] The study thus evaluated the importance of BCG reaction kinetics upon following the official recommendation. This is in contrast to most of the earlier studies, which typically included infants that had received BCG later during infancy. The study was also the first to evaluate the importance of early BCG reaction kinetics by two months – a timepoint where most BCG reactions are BCG pustules. BCG pustules likely reflect the presence of live BCG at the injection site and thus ongoing immune training. Since pustules are on average larger than the other reaction types, the strong link between BCG pustules, vaccination quality and the BCG strain might be the reason why we identified a strong association between the reaction

size and subsequent all-cause mortality. In the study by Storgaard et al, no effect of the BCG scar size was reported, but the infants included in that study were also considerably older at both the time of BCG vaccination and scar assessment.[133]

This study only included two month-old infants that were found at home, 198 had died before the visit. We were thus left with examining the survivors that were found at home. The all-cause mortality is higher earlier in life, e.g., between 0-2 months than 2-12-months. The reason for conducting the follow-up visit was to gather information regarding the first infant vaccinations provided at six weeks of age. Infants in Bissau and most other low-income countries receive a series of vaccinations at this time, namely pentavalent vaccine (DTP-HepB-Hib-1), OPV-1, PCV13-1, and ROTA-1. Since the pentavalent vaccine has been associated with detrimental effects on all-cause mortality for females in a series of observational studies, it would have been interesting to have had BCG reaction assessments performed at an earlier age. We could have then assessed the importance of having an early BCG reaction, the reaction size and type (scar, pustule or papule) and the subsequent effects on mortality *when* the infants receive the vaccinations at six weeks of age. As opposed to 2-3 weeks later, when a substantial share of deaths associated with DTP/penta would likely have already occurred.

As in the other studies with HNSM as the principal place of inclusion and vaccination, it is a strength in the study that the vaccination quality is high. Despite the high BCG reaction rate, it was possible to show an effect on all-cause mortality of having a BCG reaction and the reaction size. When compared with earlier studies, the large variation in BCG reaction prevalence across the studies makes it unlikely that the effects demonstrated are simply due to host factors among frailer infants. The strong biological correlation between the post-vaccination wheal size and the BCG strain administered and subsequent reaction kinetics further strengthens this notion. On the other hand, had the BCG vaccinators been less skilled, we would have had more non-reactors and thus more events in the non-reactor group. This might have provided power to show an effect on infectious disease mortality of reacting to BCG versus not reacting.

We included data regarding cytokine responses in a subgroup of infants that had been bled at 4-weeks of age. This was important as we were able to show that BCG reactions and the BCG reaction size at 4-weeks are associated with higher cytokine responses. There were, however, several limitations in the analysis. First, the blood samples had not been collected at the same time (four weeks versus two months of age), and not all infants that had been bled were thus visited at two months of age. Second, the main purpose of the original immunological sub-study was to collect blood samples, not to inspect BCG reactions. The personnel were thus trained to handle the important blood samples sufficiently and received supervision in these procedures, while the assessment of the early BCG reactions was given less priority.

We assumed that the infant MUAC is a mediator of the beneficial effect of BCG reactions and that developing a BCG reaction is not dependent on host factors, giving the large discrepancy in BCG scar prevalence that has been reported earlier. If infants with large MUACs tend to have larger BCG reactions (due to having physically larger arms and more

skin surface area, not because BCG had a positive effect on health and/or growth), then the infant MUAC could have been a confounder in the analysis. Our analyses were, however, robust to adjustments for factors known to affect mortality.

The RCTs that delivered the data for the analysis were conducted between 2002-18, and ten different assistants were involved in the home visits. We did identify differences in the BCG reaction assessments by assistant, which was one of the reasons why we adjusted the analysis for RCT, along with the trend of declining mortality over the years spanning the RCTs. The consistent pattern of reduced mortality associated with both having a reaction and the reaction size across the trials was reassuring. Originally, we planned to analyze the effect of different *reaction types* and subsequent all-cause mortality. Unfortunately, the prevalence of the different reaction types varied substantially between the different reaction assessors, with the most notable difference being a low prevalence in the older studies. Since our focus has traditionally been on reacting or not (scar versus no scar), this was probably comprehensible.

On the other hand, the reaction sizes had been collected more systematically, and it is also a more objective measurement to perform, rather than judging whether a BCG reaction is a pustule, a papule or a scar. BCG pustules are generally larger than papules and scars and BCG strains mainly affect the pustule prevalence, immunogenic strains being associated with more pustules and fewer papules and no difference in the scar prevalence (unpublished data available on request). It is therefore likely that our results reflect that BCG pustules are associated with lower all-cause mortality. Since we are collecting more data on this subject from both subsequent RCTs and at the Pediatric ward, it will be possible to more closely answer this question in the future.

All of the published studies that have evaluated the association between having a BCG scar or an early BCG reaction and subsequent all-cause mortality originate from Guinea-Bissau. Other groups must analyze data from other settings to assess whether our results can be reproduced.

6.1.5 Paper V

Neonatal Bacille Calmette-Guérin Vaccination and Tuberculin Skin Test Reactions at 2- and 6-Months: Effects on Mortality up to 1 Year of Age

In this study, we evaluated the effects of having received BCG during the neonatal period. The criteria were thus rather strict compared to earlier studies, but there might have at the same time been a rather short time to develop a TST response for infants that received BCG late in the neonatal period. Evaluating TSTs is important because TSTs provide an opportunity to evaluate the actual immune response induced by BCG that can be mounted by the infant after stimulation, rather than simply assessing the BCG skin reaction. There are some difficulties associated with the approach, too, however. Most notably, separate informed consent from the mother or guardian needs to be given before a TST can be applied. We noted that the subsequent mortality was quite high among infants that did not have a TST performed, indicating that the mothers might have been reluctant to give consent if the infant was sick or frail. It is also possible that the zone assistants or nurses

performing the application of TST were reluctant to do so for sick and frail infants, or a combination. Either way, we might have underestimated the effect on all-cause mortality of having a positive TST, if infants with higher mortality were less likely to have a TST performed. On a similar note, we were only capable of testing the infants that were present, meaning that infants that had died prior to the visits could not be tested. Compared to assessing BCG reactions, TSTs are more logistically complicated because a nurse has to be available to apply the TST, and a trained zone assistant has to visit the child again to read the TST result within 48-72 hours after application. In both our datasets, we were able to evaluate the TST responses within 48-72 hours for 87% of the infants that had a TST applied, and this loss to follow-up reduced our sample size. Furthermore, we had few deaths, especially for the 6-12-month analysis, which meant that the power for this comparison was reduced. Finally, it was a weakness that two different TST strengths (2 and 10 TU) had been used.

Since two different studies with slightly different designs delivered the data for the analyses, we had slightly different data available for the analysis of determinants for developing TST reactions. With complete information available for all the included infants, we would have had more power to analyze the importance of the vaccinator, the place of inclusion, and the post-vaccination wheal.

Across the studies, we had different nurses apply the TSTs, and different field assistants to conduct the TST readings. There can have been differences in the injection technique and TST assessments that could have affected our results.

Rather than conducting a simple meta-analysis of all the available data, we analyzed the effects on subsequent all-cause mortality up to one year of age by TST reaction status at 2- and 6-months of age, respectively. We did this for two reasons: first, our BCG scar data has suggested that having a BCG scar has the strongest effect on mortality if the infant was vaccinated during the neonatal period. The effect was most pronounced in the first year of life.[4] If having an early TST reaction by two months of age has a substantial impact on subsequent all-cause mortality, then it becomes an important argument for giving priority for providing BCG at birth. Second, some infants included in both the present study and in two of the previously published studies had TSTs performed at both two and six months of age. Combining the data in one meta-analysis would mean that some infants would contribute risk-time twice to the analysis. Since all studies were conducted in Guinea-Bissau using similar methodology and the heterogeneity was low, we reported the fixed-effects estimates in the meta-analysis.

As for the BCG scar studies, all studies evaluating the association between having a TST and subsequent all-cause mortality originate from Guinea-Bissau. Other groups must analyze data from different settings to test the reproducibility of our findings.

6.2 Comparison with other studies

The results outlined in Paper II indicating a substantial effect on in-hospital case-fatality among neonates that received BCG at birth complements the results of the main RCTs, supporting the conclusion that BCG-Denmark is associated with substantial beneficial NSEs.

These results are in contrast to the two large RCTs conducted in India that reported no effect of providing BCG-Russia at birth to neonates admitted to the NICU, the combined HR being 0.98 (0.85-1.11).[48]

There are important differences between the study designs that might explain this difference. The BCG strain could play a substantial role and might explain the lack of a beneficial effect. Still, given that BCG-Russia does contain *some* viable mycobacteria inducing BCG reactions to *some* extent, it would seem unlikely that there is absolutely no beneficial effect of providing BCG-Russia. This is unless other differences in the trial design also influenced the results. One such important difference is that the neonates included in the RCTs from Guinea-Bissau were vaccinated *at discharge* from the hospital, while the neonates included in the RCTs in India were vaccinated upon admission at two different NICUs in India. Inclusion criteria were furthermore different since neonates weighing below 2.500 kilograms were included in the Bissau RCTs, while only neonates weighing below 2.000 kilograms could be enrolled in the Indian RCTs. The neonates were slightly younger in the Indian RCTs (median one day) versus the Bissau RCTs (median two days in both large-scale RCTs).[8,48,140]

The neonates enrolled in the Indian RCTs were thus substantially more vulnerable, which is well illustrated by the overall neonatal mortality rates of 15.8% (487/3,072) in the first trial and 17.8% (392/2,207) in the second trial. This is to be compared to an overall neonatal mortality rate of 2.9% (189/6,544) in the RCTs from Bissau. There are likely deaths that BCG cannot prevent; it has not previously been shown that BCG can prevent deaths due to prematurity, asphyxia, or birth complications.

A separate issue is that neonates admitted to the NICU might be in a more stressed state where inflammatory immune responses would already be activated to a larger extent. If BCG principally works by stimulating similar pathways, then it would be understandable that additional stimulation of the same pathways has little or no additional effect. Even more so when the stimulation provided is also relatively weak, given that a less immunogenic strain of BCG was utilized.

We have conducted an RCT in Bissau randomizing neonates admitted to the NICU at the maternity ward of HNSM to BCG+OPV immediately upon admission versus BCG+OPV at discharge, and we are currently preparing the data for publication. The strains used for that trial were BCG-Denmark and BCG-Japan. Importantly, the authors of the Indian RCTs are also conducting a new RCT comparing BCG-GreenSignal versus control.[128] There will thus be more information available regarding the usefulness of providing BCG also to the frailest neonates admitted to the NICU, and whether there are strain differences in these effects.

In the data presented in paper III, we had reduced power, especially for the mortality comparison, as discussed. It is nevertheless odd that we did not identify a tendency of lower mortality associated with BCG-Denmark when compared to BCG-Russia but rather the contrary, while we did see a tendency of reduced mortality associated with BCG-Japan.

For papers IV and V, our data corresponds well with the previously published studies evaluating BCG and TST reactions. The combined data thus presents a strong argument for

ensuring the provision of BCG at birth administered by well-trained vaccinators. The use of immunogenic strains such as BCG-Denmark and BCG-Japan is to be encouraged, as it would increase the BCG reaction prevalence, the average reaction size and the prevalence of TST conversions.

6.3 Interpretation and perspectives

The combined epidemiological and immunological evidence from Guinea-Bissau and other countries strongly suggests that BCG has beneficial NSEs. BCG thus has the potential to reduce mortality substantially already early in the neonatal period through the induction of EG and trained immunity, but it can also be used to combat bladder cancer and possibly lung cancer, as indicated by recently published data.[151]

The relative effects of BCG strains are important too, and it should become one of the main focuses when it has been assured that BCG is provided to vulnerable populations as close to birth as possible. Finding the best BCG strain and increasing production and delivery of this strain would naturally be the next steps, but is easier said than done. Determining the importance of parental immune priming with BCG is also of paramount importance and could become a game-changer for the use of BCG.

There has been a remarkable drop in overall childhood mortality in Sub-Saharan Africa in the last decade.[176] However, a similar decline has not been registered in neonatal mortality.[177] In 2016, 2.6 million deaths occurred in the neonatal period corresponding to 46% of all deaths under five years of age.[178] On current trends, more than 60 countries will miss the Sustainable Development Goal by 2030 of reducing neonatal mortality to fewer than 12 neonatal deaths per 1,000 live births.[178] Neonatal deaths thus represent an increasing share of the total deaths, and sepsis affects an estimated 3 million newborns per year.[179] Three out of ten deaths from neonatal sepsis are believed to be caused by pathogens that are resistant to antibiotics.[180] In a large study of early-onset neonatal sepsis (EONS) in Soweto, South Africa, 1,231 newborns met the criteria for protocol-defined EONS.[181] Despite broad testing for a series of possible pathogens, only 27% (329/1,231) of cases with protocol-defined EONS had attributable etiology, and blood cultures were only positive in 8% (99/1,231).[181] Among those with positive cultures, a range of pathogens were responsible, including group B streptococci, V. streptococci, enterococci, S. aureus, E. coli, A. baumannii, H. influenza, N. meningitides, and S. paucimobilis. Targeting each of these pathogens independently with vaccines is not currently feasible for many reasons, including the lack of available vaccines and the weeks needed to mount an adaptive immune response. Providing vaccines during pregnancy and relying on placental transfer of antibodies might have potential, but it would require stringent safety testing and raises a series of concerns. Given the wide range of pathogens responsible for neonatal sepsis and the possibility of *pathogenic drift*, e.g. that other pathogens might take over if we manage to prevent some of the pathogens causing sepsis, it seems that a different remedy is needed. A remedy that provides a broad, imminent non-specific boost of host immune defenses towards a range of pathogens, such as BCG.

Furthermore, if parental immune training with live vaccines has substantial benefits, then the implications are substantial. In Guinea-Bissau, the maternal BCG scar prevalence varies

substantially by age (among other things due to fluctuations in the national immunization schedule and a damaging civil war in 1998-99). The overall prevalence among mothers currently giving birth at HNSM is about 64%, but it is lower in the younger and the older mothers (unpublished data). If maternal and possibly also paternal immune priming with BCG has substantial effects on the offspring, and one wishes to further enhance these effects, then >1/3 of the adult population in the fertile age should potentially be vaccinated. Given the disparity in the maternal scar prevalence across maternal age distributions, there are perspectives in going back in the epidemiological data and assessing the impact of the overall maternal BCG scar prevalence on infant outcomes over time. For example, we could assess the influence of maternal BCG prevalence on the LBW RCTs over time, to examine whether a high average maternal BCG vaccination coverage versus a low coverage is associated with better infant outcomes. The child mortality has been declining in Bissau over the last decade, and the prevalence of maternal BCG scars has been increasing.

Since we initiated the RCT comparing strains in 2014 (reported in paper III), the field of BCG research has moved forward with many lengths. Many groups are now investigating BCG's effects and properties from many angles. New papers are published at a steady pace. When it comes to BCG, the concept of NSEs appears to have become rather mainstream. This is timely, because if these important effects are not described, debated and appreciated, then a new efficient anti-TB vaccine might be licensed, and BCG could disappear from vaccination programs. Unless such a new vaccine is also a live-attenuated vaccine with marked beneficial NSEs, such a switch could lead to a public health catastrophe.

BCG still deserves a much better reputation, however. The vaccine both has beneficial NSEs and it *does* protect against the more severe forms of TB. It seems that just because it is an old and perhaps simple or primitive vaccine that results in clumsy pustules, it is not prestigious or trendy. But things seem to be changing on that front. For example, BCG will be provided to health-care professionals in rushed RCTs in the Netherlands and Australia with the hypothesis that vaccination can provide front-line medical workers much-needed protection against COVID-19.[182–184] There is thus plenty of enthusiasm surrounding BCG, and for good reasons. Humanity needs a tool to boost the immune system and reduce the burden of infectious diseases. The world population is estimated to grow to approx. 11 billion by the end of the 21st century.[185] Urbanization is a global megatrend that will increase the share of city-dwellers from 55% in 2018 to 68% in 2050 – up from 30% in 1950.[186] This will likely result in improved conditions for the spread of infectious diseases, human competition for resources, inadequate provision of health care and increased indiscriminate antibiotic use. We are already on the brink of a massive crisis due to bacterial resistance to commonly used antibiotics. The poor, the young, the elderly, and other weak groups will likely suffer the toughest consequences. In such a situation, we ought to make use of the advantages of BCG vaccination to boost innate immunity and also make the best use of other live vaccines such as MV, OPV, and perhaps even smallpox vaccination. Aside from strengthening the body's defenses against infectious diseases, BCG seems to lower the level of steady-state inflammation in the body (unpublished data). If this is the case, then BCG might also affect autoimmune and allergic diseases (which has boomed since BCG was discontinued in high-income countries and is uncommon in low-income countries). One

study thus reported an effect against type 1 diabetes mellitus and an RCT is being conducted[187], an observational study indicated protective effects against Alzheimer's disease among patients that received BCG against bladder cancer[152], while an RCT has indicated that BCG reduces the cumulative number of CNS lesions after the first demyelinating event among patients with multiple sclerosis.[188]

6.4 Immediate and long-term policy consequences

The notion of NSEs related to BCG has become more mainstream; more scientists are engaging in this research area. The appearance of several mechanisms – most notably EG and innate immune training – has shown that there are plausible immunological pathways through which the effects are mediated. The acknowledgment of marked beneficial NSEs associated with BCG in the WHO 2014 review created hope that this would create more focus also from authorities and stakeholders on the NSEs of both BCG, DTP, and MV. Unfortunately, little has happened when it comes to real-life changes. Despite the importance of high vaccination coverage for BCG to exert both its specific and non-specific effects, the administration of BCG is unfortunately often delayed due to a host of logistical factors and inherent health care system shortcomings.[189,190] Providing BCG at birth is not a priority in health care programs in LMICs. Also, WHO reports BCG coverage by 12 months of age, rather than reporting the neonatal BCG coverage. In the most recent estimate of the BCG vaccination coverage in Sub-Saharan Africa published in 2009, neonatal coverage was below 50%.[191] In a study of 5,171 infants from community-based BCG clinics in Lagos, Nigeria, 31.6% (1,634/5,171) had not received BCG by 3 months of age.[192] Undernourishment, lack of antenatal care, and multiple gestations were predictive of delays beyond six weeks of age, indicating more frequent delays among the more vulnerable neonates. There is thus a substantial potential for public health in optimizing the use of BCG, because BCG is not provided in high-income countries and is often delayed in low-income countries. Separate considerations would apply as to whether it would be recommendable to reintroduce BCG in high-income countries, given the limited supply of BCG globally and the relatively moderate importance of its beneficial effects when compared to the effects in low-income countries. The infants that need BCG most often do not receive it. For example, Guinea-Bissau currently experiences a stock out of BCG, which has lasted for 5-6 months and a stock out of OPV that has lasted almost one year. If not for the BHP RCT currently comparing BCG-Japan versus BCG-Russia, neonates would not receive BCG at the maternity ward. This is a major public health issue that has to be resolved.

It is thus urgent to remove barriers to vaccination, including also the restrictive vial policy, which is not even based on the actual number of doses in the BCG vials, as demonstrated in paper I. Another important aspect that was recently carefully described by my colleague, is the household costs associated with procuring vaccination. In Bissau's rural areas, mothers bring their infant for BCG vaccination 1.3 times on average, with an average cost of 1.9 USD per BCG-vaccinated infant.[193] Some 42% of mothers had brought their infant for BCG vaccination in vain. Not only did this deprive the infants of important health benefits, it also means that any vaccination program savings caused by the restrictive vial policy are transferred as costs to the families.[193]

Assuring that BCG is provided at birth to as many neonates as logistically and economically feasible should be the top priority given the immediate and long-term effects demonstrated.

RCTs comparing NSEs of BCG-GreenSignal (derived from BCG-Denmark) versus BCG-Russia versus no BCG are being planned in eastern Africa, and an RCT comparing BCG-GreenSignal versus no BCG is underway in India. It might thus be possible within the foreseeable future to convincingly establish whether there are marked effects associated with different strains on hard clinical endpoints like all-cause mortality. Effects on BCG reaction prevalence, reaction sizes, and TST responses might not convince all stakeholders. Some might believe that these are "false" correlates of protection that are unrelated to clinical outcomes, as proposed by George Comstock.[195]

Hopefully, the combination of an increased acknowledgment of BCG's NSEs and the accumulation of data indicating strain differences will increase the focus on providing immunogenic BCG at birth and eliminate all obstacles for early vaccination. The next challenge will be to convince largely political organizations such as the WHO to prioritize both early BCG and immunogenic strains. Should that be successful, there will be challenges associated with the production of the different BCG strains since BCG-Russia currently represents a considerable 41% (77m/185m) share of the BCG vaccines procured via UNICEF in 2018, as detailed in Table 1. In case the genetically identical BCG-Bulgaria can be regarded as equivalent to BCG-Russia, then a combined 61% (114m/185m) of the current BCG vaccines might need to be substituted. Steps should thus be taken carefully because receiving a weak BCG vaccine is better than receiving no vaccine. A feasible and ethically sound approach might be for UNICEF to limit the distribution of superior strain(s) to areas, populations, or countries with high neonatal mortality and a high TB incidence. This would ensure that the BCG strains would be used where they have the greatest impact, provided that non-specific and specific effects related to BCG are correlated. Another possibility would be to request producers to increase the content of viable mycobacteria in strains such as BCG-Russia, or to increase the dose administered, and test whether equivalent effects are achieved in this manner. Such steps would, however, likely limit the total BCG production output.

6.5 Future research questions and study ideas

The importance of the dose of BCG has not been studied satisfactorily. The current dosage recommendation is mostly based on the wish to reduce side-effects and was mainly pushed by the Danish manufacturer, which happened to produce the BCG strain most often associated with side-effects such as lymphadenitis. It would be important to further study whether a higher dose could provide better specific and non-specific effects, and at which relative cost in terms of adverse-event prevalence. We could apply more simple study approaches. For example, different BCG doses or BCG strains could be supplied on even and uneven months or years and the subsequent all-cause mortality and TB incidence evaluated with relatively low risk of bias, especially if several sites participate.

Should BCG strains be administered in different doses due to their inherent differences? Or should producers of weak strains be petitioned to increase the CFU content in their product

to a standardized level? The RCTs from India used two different doses of BCG-Russia, but both had no effect.[48]

In Bissau, our group will continue to investigate BCG strains. A second BCG strain RCT in Guinea-Bissau is due to be finished in September 2020, comparing >15,000 infants randomized to BCG-Japan versus BCG-Russia.[125] A third trial protocol is being submitted for ethical review to initiate a trial of similar size. An important trial would be a comparison of providing a booster-dose of BCG with the third dose of pentavalent vaccine at 14 weeks of age versus no-booster. Providing BCG at this time would likely help remedy the detrimental NSEs related to pentavalent vaccination.[198] In a just-published analysis, professor Shann estimates that more than 1 million lives could be saved in a live-vaccine-last vaccination schedule.[199]

Since we have found indications of beneficial effects related to maternal BCG vaccination, an interesting study to perform would be to randomize women in the fertile age with a negative pregnancy test to receive BCG or placebo. Subsequently, their future offspring could be randomized to receive BCG or placebo. Such a study would bring forward substantial about the interplay between the maternal and infant immune system and could have a remarkable impact. It would not be feasible or ethical to conduct the study in an African setting (e.g. Bissau) because all neonates should receive BCG at birth and almost two-thirds of the mothers have a BCG scar. But it could be conducted in Denmark where practically no women in the fertile age will have received BCG and where BCG-at-birth is not standard practice.

The importance of BCG skin reactions and TST responses for NSEs are quite clear, but are they also good correlates of protection against TB? In a few years time, it should be possible to conduct additional follow-up in the BCGSTRAIN I and II cohorts. Such efforts could reveal interesting data regarding the protection against TB induced by different strains, BCG reactions and TST responses.

Finally, the global pool of BCG strains could be used more intelligently. The data linking BCG scars and TST responses with enhanced survival is unequivocal and so is the association with the strain administered. It should therefore be considered shipping the immunogenic strains to regions with high mortality indices, which would likely also coincide with regions with high TB incidence, and sending the less immunogenic strains to regions where the expected impact of BCG is less substantial.

6.6 Conclusion

Our findings confirm that BCG strains have beneficial NSEs since BCG-induced skin reactions and TST responses were associated with enhanced subsequent survival. Receiving early BCG-Denmark dis not affect the incidence of hospital admissions in Guinea-Bissau, a setting with relatively high mortality and infection pressure, but reduced the risk of in-hospital neonatal death, with special mention to neonatal sepsis.Hence, BCG perhaps affect severity of infection more than susceptibility to infection. We conducted a large-scale RCT which was underpowered for the comparison of all-cause mortality related to the different strains, but we did note a tendency towards fewer deaths associated with BCG-Japan when compared to BCG-Russia and are conducting a follow-up RCT to pursue this finding. Since receiving BCG-Japan and BCG-Denmark rather than BCG-Russia was also associated with better BCG reaction and TST responses, those strains are likely more immunogenic. Due to the amount of BCG vaccinations performed each year which exceeds 120 mill. doses, these findings could have a substantial impact on public health. It is, however, premature to call for a phase-out of BCG-Russia. Since all BCG strains tested did produce some degree of BCG reactions and TST reactivity, it would be fair to assume that all are associated with some degree of beneficial NSEs. BCG-Russia is currently an important pillar in the global efforts to provide early BCG to at-risk populations and receiving a weak BCG vaccine is probably better than receiving none. More data is thus needed to draw definitive conclusions about strain efficacy – and this goes for both their specific and non-specific effects.

6.7 Philosophical afterthoughts

Within research and in life in general, remaining humble, receptive, and bringing constructive criticism are cherished human values. It is not rare that the most remarkable advances in science happen through sheer coincidence. Rather than dismissing extraordinary findings as *biologically implausible*, we should seek to reproduce these findings and try to test alternative hypotheses. We should as scientists champion the unexpected findings rather than only having faith in findings that were planned a priori and thus predicted or known before the study had begun. We should react with balanced levels of criticism and curiosity when the data seemingly does not fit the dominating paradigm.

We have to test our hypotheses in the data and not simply rely on antiquated hypotheses and business as usual. Guidelines or recommendations that were implemented without testing the consequences, as is the case for several vaccines that have largely never been tested for their overall health effects in randomized trials, will have to be scrutinized. After having worked with NSEs since 2012 and seeing the development that has happened, I am optimistic. I believe there will come a day where we test thoroughly and with sound methods whether our vaccination programs can be improved substantially, taking NSEs into account.

Many important discoveries through human history, like that a certain fungus could destroy cultures of staphylococci and a range of other bacteria, were completely unexpected and unplanned. Alexander Fleming famously remarked, "*That's funny!*"[200] and:

"*One sometimes finds, what one is not looking for. When I woke up just after dawn on September 28, 1928, I certainly didn't plan to revolutionize all medicine by discovering the world's first antibiotic, or bacteria killer. But I suppose that was exactly what I did.*"[201]

Other examples include the discovery of heparin, X-rays, the Gram staining technique and the role of the pancreas in diabetes. The majority of landmark discoveries within biology and medicine have had a serendipitous element in them.[202]

Serendipity in research is thus a real and important concept, and the discovery of NSEs in Guinea-Bissau was certainly not planned. Instead, it was strongly opposed to the dominating ideas in the medical community of the time and still is to some extent. It was also a serendipitous coincidence that follow-up visits by three days of age were conducted to evaluate early adverse events in the LBW BCG trials. This led to the finding of the remarkable effect on mortality already by three days after randomization. Should we dismiss such an observation, just because it was not a planned or expected finding? Had the effect not been reported – or had it been dismissed by the entire scientific community as it was dismissed by some, then the concept of EG and many other remarkable effects associated with BCG might have never been discovered.

Similarly (but much less remarkable): I vaccinated my daughter, wife and parents with BCG-Japan. We all developed large, nasty pustules that took many months to heal. This observation led to the idea that large BCG reactions and BCG pustules might be beneficial and were thus the serendipitous element of the data presented in Paper IV.

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8 Appendix

8.1 Overview of other modes of BCG administration

Aside from the oral and intradermal administration routes, a percutaneous, multi-puncture technique was developed in Japan to reduce adverse events following vaccination (Figure 43).[1] The technique has been used in Japan, the UK, South Africa, South Korea and in Brazil.[203]

Figure 43. Multi-puncture device (Kuchiki's needle) with nine short needles shown with a BCG ampoule and saline vial used for BCG vaccination. Photo credit: Y. Tambe (no changes made). License: Attribution-ShareAlike 3.0 Unported (CC BY-SA 3.0) https://creativecommons.org/licenses/by-sa/3.0/

A drop of the reconstituted vaccine is spread over an area of approx. 1.5 cm by 3.0 cm, and several small punctures are applied using the multi-puncture device in order to vaccinate using this method.[89] This technique is easier to administrate for the vaccinator, but it also implies using larger amounts of resuspended BCG, since not all of the vaccine is delivered in the skin, and it is difficult to access the final dose delivered.[89] The concentration of reconstituted vaccine for intradermal use is 1 mg/ml in the 0.05 ml dose. In contrast, the percutaneous vaccine is made up of 1.0 ml BCG at a concentration of 40 mg/ml, or 800 times as much.[89] Vaccine wastage is thus a separate, substantial concern with this method. A small observational study has compared percutaneous BCG-Japan with intradermal administration of BCG-Japan and BCG-Denmark. The results indicated that percutaneous BCG-Japan was superior to intradermal BCG-Denmark.[204] However, a large RCT from South Africa that included 11,680 infants randomized to receive BCG-Japan delivered either by the percutaneous route versus the intradermal route found no differences in the cumulative incidence of TB, adverse events or deaths.[205] Interestingly, however, the mortality was 1.73% (102/5,905) for infants vaccinated intradermally versus 1.45% (84/5,775) for the percutaneous route, the intradermal/percutaneous RR being 1.19 (0.89-1.58).[205] The results from South Africa have been corroborated by a study from South Korea that reported no differences in the immune responses associated with BCG-Pasteur delivered intradermally versus BCG-Japan delivered using a multi-puncture device.[206] A study in school-children from the UK reported fewer Heaf test (a TST test delivered by use of a multi-puncture device called a Heaf gun) conversions, fewer visible BCG scars and less adverse events associated with percutaneous BCG versus intradermal BCG.[207]

There has recently been renewed interest in administering BCG by the mucosal route (oral or intranasal delivery)[45]. A study in rhesus macaques has indicated that pulmonary mucosal BCG vaccination followed by TB challenge confers reduced local pathology and improved hematological and immunological parameters when compared to intradermal vaccination.[208] Similarly, a study has indicated that providing intranasal BCG to mice induced better protection than intradermal administration.[209] A Brazilian study on guinea pigs compared oral BCG-Moreau to subcutaneous BCG-Denmark in an aerosol infection

model of pulmonary tuberculosis. The study indicated that oral BCG-Moreau provided protection equivalent to subcutaneous BCG-Denmark and was associated with statistically lower bacterial loads in the lungs and spleens.[210] The authors argued that oral delivery has substantial advantages since needles (and associated cross-infection risks) and specially trained medical staff to deliver the difficult intradermal vaccination is avoided. It is problematic, however, that different vaccine strains and delivery techniques were tested at the same time in several of these studies since it is not possible to disentangle whether differences were due to the strain provided or the method of vaccine delivery.

Oral vaccination would likely reduce the costs of vaccination since single-use needles would be avoided, and the vaccine could be administered more easily with less need for specialized training, as is the case for OPV. Since TB is spread via the mucosal route, the implications of intranasal, intrabronchial, and oral administration of BCG merits further evaluation. Further evidence of the possible role of the administration method was highlighted in a recent study that reported boosted protection against mainly pulmonary TB in macaques given intravenous BCG when compared to intradermal and aerosol delivery.[211] The study included a mycobacterial challenge with virulent *M. tuberculosis* six months after BCG vaccination, from which nine out of ten macaques that received intravenous BCG were protected. Such effects might reflect increased access to the bone marrow when BCG is administered intravenously, and the importance of the possible routes of administration certainly deserves further study. Providing BCG by the intravenous route has notable drawbacks for mass vaccination, though: substantial specialized training vaccinators and enhanced safety measures would have to precede the implementation of broad-scale intravenous BCG administration.

8.2 The importance of maternal immune priming

A series of Ugandan immunological studies have linked maternal BCG scars with effects in the immune response of the offspring.[51,212,213] In Bissau, the importance of maternal priming was first evaluated for measles vaccination. This is because one of the main arguments to not provide MV earlier than nine months after birth in low-income countries (and 15 months in Denmark) has been the assumption that the vaccine would be less efficacious if administered under the presence of maternal antibodies in the infant. Interestingly, vaccination under the presence of maternal antibodies has been associated with lower all-cause mortality than being vaccinated in their absence.[214] Due to this finding, it was prespecified in a Danish RCT of BCG-Denmark versus no BCG to analyze the trial data by maternal BCG vaccination status. The hypothesis was that receiving BCG would be more beneficial if the mother had been BCG vaccinated.[194] Overall, BCG did not prevent hospital admissions due to infectious diseases in the study, the HR being 0.99 (0.85-1.15). Among the 17% (740/4,262) of infants that had a BCG-vaccinated mother (based on maternal recall), however, being randomized to BCG versus no BCG was associated with an HR of 0.65 (0.45-0.94) for infectious disease admissions, while it was 1.10 (0.93-1.29) if the mother was unvaccinated.[194]

These findings led to the study mentioned above indicating that maternal BCG scarring modulates the NSEs of BCG scars in the offspring.[134] In an RCT from India that evaluated

BCG-Russia versus no BCG, however, there was no effect of BCG-Russia on the in-hospital mortality, and maternal BCG scars also did not affect the BCG-Russia versus control mortality ratio in the trial.[48] While it remains to be investigated whether the importance of maternal BCG priming is dependent on the BCG strain provided to the offspring (and/or to the mother originally), there are thus indications both from high- and low-income countries that maternal BCG vaccination can modulate the immune response to BCG in the offspring. A mechanism for vertical transmission of BCG from vaccinated mothers has also been proposed.[215]

These findings have very important implications as maternal BCG scarring might have been a major confounder in previous BCG studies. For example, the protective effect of BCG has been greater in trials conducted at latitudes farthest from the equator. The consensus has been that this is likely due to a higher presence of environmental mycobacteria closer to the equator, which has been assumed to lower the protective effect of BCG against TB.[2] A competing hypothesis might be that BCG vaccination had begun earlier and was more prevalent in areas further from the equator (e.g., Europe, North America, South Africa, Southern parts of South America). Mothers to the infants studied were therefore more likely to have been BCG vaccinated.

Another separate and even less studied factor of possible importance is paternal immune priming. When the data from the Danish RCT in Bissau became available, a scar recognition course was held for the inclusion and follow-up assistants of the studies being conducted at the Maternity Ward. The purpose was to train the personnel working on the studies to recognize maternal BCG scars and to differentiate these from smallpox vaccine scars and common scars due to cuts and scratches, etc. The data collection on maternal BCG scarring began at the time of inclusion at the Maternity Ward in the trials conducted there from July 2015 (Figure 44A, B). These important data lie beyond the scope of this Ph.D. dissertation.

Figure 44, A+B. Assessments of maternal scars at the HNSM Maternity Ward in Bissau. Photo credits: A: Rebecca Alison Fabricius. B: Sofia Busk.

8.3 Potential immunological mechanisms behind BCG's beneficial NSEs

8.3.1 Immediate effects of BCG on granulopoietic capacity mediated by induction of G-CSF: novel insights from a murine sepsis model, human transcriptional signatures and epidemiological data (RCT I-III)

The first RCT evaluating BCG versus no BCG to LBW neonates was planned to investigate the effect of BCG on infant mortality (up to one year of age).[140] Since follow-up visits were conducted at three days after randomization to evaluate BCG-associated adverse events and also at 2-, 6- and 12-months of age, the effect on mortality of receiving BCG versus control was also reported at these time points.

Surprisingly, BCG almost halved the mortality rate already by three days after randomization, the MRR being 0.55 (0.32-0.93), and the estimate by 28 days of life was an MRR of 0.62 (0.46-0.83).[8] The effect by three days was more pronounced for males, for whom mortality was reduced almost 3-fold, the BCG/control MRR being 0.36 (0.20-0.67).[216]

There were a total of 73 neonatal deaths in the BCG arm and 116 deaths in the control group, but a substantial share of the absolute mortality difference (43 deaths) between the two groups had occurred already within the first three days after randomization.[8] By three days after randomization, there had thus been 22 neonatal deaths in the BCG arm and 39 deaths in the control arm. From day four to day 28 after randomization, a further 51 BCG and 77 control neonatal deaths occurred. Since there were two BCG neonatal deaths and eight control neonatal deaths on day four after randomization, the difference from day five to day 28 of life was even less (49 BCG deaths versus 69 control deaths). This provides indications of several important aspects of BCG's beneficial effects when provided at birth:

First, as was also previously reported, a substantial share of the total neonatal effect seems to have occurred quite rapidly after randomization, e.g., within 3-5 days, indicating rapid beneficial effects on the innate immune system induced by BCG.

Second, very few would be BCG vaccinated in the control group within 3-5 days after randomization, since it was not the national policy at the time to provide BCG to infants weighing under 2.5 kilograms. Most would thus be barred from vaccination given that the infant had been below the weight cut off a couple of days earlier. As such, the comparison by 4-5 days of age might provide a more *clean-cut* comparison since RCTs are only truly randomized just after randomization.

Third, if BCG induces a swift beneficial immune response within days of vaccination, it becomes very interesting to elucidate the immunological mechanism.

When the rapidly induced beneficial effects of BCG on mortality were first presented in the large RCT from Bissau published in 2011, the results were, however, met with skepticism and disbelief. Quite prominent researchers argued that it had to have been due to lack of blinding and methodological flaws in the study, such as an inherent reluctancy by the BHP staff to vaccinate frailer neonates, effectively placing those with the highest risk of death in the control group.[217] It was noted that the reduction in mortality occurred entirely in the

first 21 days of life and the critics went as far as to claim that the results of the trial were "*hard to explain in terms of immunology or physiology but may be plausibly explained in terms of a procedural flaw."*[217] This is despite there being no differences between the randomization groups in relevant nutritional indices such as weight and other risk factors. In fact, there were more twins in the BCG arm and a tendency towards more perinatal maternal deaths in the BCG arm, meaning that the distribution of baseline factors actually tended to be in favor of the control group.[218] Also, the finding of a beneficial effect on mortality within three days of randomization was a replication of the results of the first small RCT conducted in Bissau, in which the MRR by three days was 0.17 (0.02-1.35).[141]

When novel and potentially paradigm-changing studies appear in medicine and science as general, it is natural to be skeptical and critically evaluate whether the science behind it is sound. It is, however, very important to maintain a humble approach and accept that the science is ***not*** settled. There are many aspects of immunology and physiology that we still cannot explain, let alone understand. The mechanisms behind extraordinary effects often only come to light later, when very deliberate and technologically advanced studies have been conducted. To state that results are essentially *biologically implausible* puts one at risk of subsequent embarrassment, especially when the novel data was published from a small group of researchers that have no particular reason to favor BCG. The motivation of the study is a strong wish to evaluate interventions and organize health measures so that beneficial effects on public health are optimized.

Due to the remarkable rapidly BCG-induced effects on all-cause mortality reported in the RCTs from Bissau, a Canadian group took up the challenge of investigating the immunological mechanisms that could explain how BCG could induce such rapid protection. In a murine model of neonatal polymicrobial sepsis, BCG induced a spike in granulocyte colony-stimulating factor (G-CSF). This fuelled an emergency granulopoiesis (EG) response leading to a dramatic increase in neutrophil counts.[147] Higher neutrophil numbers were then directly and quantitatively responsible for drastically improved protection from sepsis among BCG-vaccinated mice versus controls in the murine sepsis model. BCG-induced EG reduced the bacterial load in the blood, spleen, liver, lung and peritoneal wash. The importance of neutrophil numbers for protection was shown by adoptive transfer of neutrophils from BCG-vaccinated versus control mice splenocytes to BCG-naïve mice. Protection was conferred in the murine sepsis model if neutrophils from BCG-vaccinated mice were transferred, but not if neutrophil numbers had been depleted before the transfer. Neutrophils were thus necessary and sufficient for protection.

Interestingly, protection against death was time-limited, and neonatal BCG vaccination thus did not confer protection when the challenge was delayed by 10-12 days, nor did vaccination of adult mice confer protection. Transcriptional signatures consistent with induction of EG were detectable in the blood of BCG-vaccinated neonatal mice as well as human vaccinated versus unvaccinated neonates from Guinea-Bissau.[147] The researchers furthermore observed that among healthy newborns in The Gambia, absolute numbers of neutrophils subsequently declined over the first week of life, and the rate of this decline was reduced among BCG-vaccinated newborns. Interestingly, total neutrophil numbers started

increasing between day one and two after BCG vaccination. First, the researchers observed an increase in immature neutrophils peaking at day two followed by an increase in mature neutrophils beginning on day two post-vaccination, peaking at day three, and returning almost to control group levels by day four.[147]

A previously published study by Ladisch et al. from 1978 offers an independent verification of this mechanism. Mice that were given BCG 8 days prior to induction of leukopenia had accelerated granulocyte recovery indicating that BCG may have clinical value as a stimulator of myelopoiesis.[219] Newborns, and particularly premature and VLBW newborns, are susceptible to neutropenia. The degree and duration of neutropenia is correlated with the development of new infections and impacts survival from various diseases including sepsis.[220–222] It is thus plausible that induction of granulocytes in the bone marrow and a subsequent spike in neutrophil numbers in the blood and spleen provide protection against sepsis. this mechanism could thus be responsible for BCG's rapid effects on all-cause mortality. It should then be possible to identify similar signals, e.g., a substantial reduction in all-cause mortality and cases of fatal sepsis approximately around three days after vaccination, in the epidemiological data from Guinea-Bissau. *A priori*, one might expect that a peak in neutrophil numbers occurring approximately three days after vaccination would translate to fewer deaths and cases of sepsis on day three and a few days later. It should be noted, however, that some inherent uncertainty related to the accuracy of the day of death and day of hospital admission data from Bissau exists. The mortality data is typically updated retrospectively during later home-visits, and neonates hospitalized during the evening or night might only be registered the day after. With this and the *post hoc* character of this analysis in mind, it indeed seems to be the case that BCG-induced protection from sepsis and death was strongest around day three to five after randomization (Table 17 and Table 18).

Table 17. Neonatal BCG versus control MRR by age during the neonatal period within selected timeframes after birth.

	Neonatal period		Day 0 to 2		Day 3 to 5		Day 6 to 28	
	BCG	Control	BCG	Control	BCG	Control	BCG	Control
Deaths	73	116	13	20	1	12	59	84
BCG/Control MRR	0.62 (0.46-0.83)		0.64 (0.32-1.28)[1]		0.08 (0.01-0.64)		0.69 (0.50-0.97)[2]	

[1]0-2 days vs. 3-5 days, p for same effect=0.07. [2]3-5 days vs. 6-28 days: p for same effect=0.04.

Table 18. Neonatal BCG versus control hospital admissions and in-hospital deaths within selected timeframes after randomization.

	Neonatal period		Day 0 to 2		Day 3 to 5		Day 6 to 28	
	BCG	Control	BCG	Control	BCG	Control	BCG	Control
All-cause admissions	84	86	18	20	17	17	49	49
Fatal admissions[1]	18	32	8	12	3	7	7	13
BCG/Control RR	0.58 (0.35-0.94)		0.74 (0.40-1.39)		0.43 (0.13-1.39)		0.54 (0.23-1.23)	
Sepsis admissions	41	54	9	14	7	13	25	27
Fatal sepsis admissions[1]	7	20	3	7	0	5	4	8
BCG/Control RR	0.46 (0.22-0.98)		0.67 (0.23-1.93)		-		0.54 (0.19-1.57)	

[1]Admissions that occurred within the given timeframe can have been fatal a couple of days later, which is why numbers might not necessarily add up in comparison to the total mortality numbers in Table 17.

The data presented in Table 17 and Table 18 does not provide a definitive answer as to whether EG was the main mechanism behind BCG's beneficial NSEs. But there were marked BCG-induced effects between days three to five on both all-cause mortality, fatal all-cause admissions, and fewer cases of sepsis admissions and reduced risk of in-hospital death due to sepsis. The epidemiological data is thus supportive of the interpretation that EG is an important mechanism behind BCG's beneficial neonatal NSEs since neonates are vulnerable to perinatal neutropenia.[222,223] The human BCG versus control transcriptomic data and the mouse mechanistic studies demonstrate that BCG acts as a strong granulopoietic inductor which could limit the vulnerability from neutropenia. If this is the case, then BCG would likely induce wide protection from various pathogens and save neonates, as was found in the three LBW RCTs. Since mortality is highest on the day of birth and then declines during the first week of life and then declines further during the first month of life, providing BCG as early as possible after birth could enhance the beneficial effects of BCG's granulopoietic properties.

8.3.2 The effect of BCG on immune responses against unrelated pathogens and on subsequent vaccine-responses

Aside from the novel study mentioned above that highlights a possible mechanism behind BCG's beneficial effects, landmark research into *trained immunity* has provided important new insights into BCG's effects on the immune system and provides plausible mechanisms to explain the effects reported from low-income countries.

In one landmark study, Kleinnijenhuis et al demonstrated for the first time that trained immunity is present in humans, and that it is induced by BCG.[9] Trained immunity had previously been documented to affect the immune systems of plants, invertebrate animals, and mice.[9] When BCG was provided to healthy volunteers, there was a four- to sevenfold increase in their IFN-γ production and a twofold enhanced release of monocyte-derived cytokines in response to unrelated bacterial and fungal pathogens. This enhanced function of monocytes was present for at least three months after vaccination and was induced through the NOD2 receptor.[9] Finally, the study tested whether BCG vaccination protects against disseminated candidiasis in mice with severe combined immunodeficiency. All BCG-vaccinated mice survived versus 30% of control mice. The finding that BCG provides non-specific protection against candidiasis in immuno-deficient mice had also been reported in studies from 1985 and 1992.[224,225]

The Kleinijenhuis study was crucial both for the field of *trained immunity* and for research into NSEs of BCG. The study demonstrated a mechanism through which BCG could exert its beneficial effects on the immune system by functionally reprogramming monocytes to an enhanced, long-lasting phenotype. The authors subsequently demonstrated that the strengthening of the innate immune system's activity against unrelated infection is long-lasting.[226] The growing body of evidence of BCG's engagement of the immune system which enhances immune responses to a broad range of pathogens raises the possibility that a substantial part of BCG's clinical benefit, and if not most, is due to beneficial NSEs.[227] These effects are remarkable because not only does BCG strengthen the responses to unrelated infections, BCG also increases antibody responses to unrelated vaccines. This has been demonstrated for polio vaccine[228], pneumococcus, *Haemophilus influenzae* type B and tetanus toxoid vaccines[229] and hepatitis B vaccine in both mice[230] and humans.[231] In a randomized study, Dutch volunteers received either BCG (n=20) or placebo (n=20) and influenza vaccine two weeks later. Antibody responses were significantly enhanced among BCG-vaccinated subjects, and there was a tendency of increased seroconversion associated with BCG (85% for BCG-vaccinated subjects versus 65% for the placebo group, p=0.08).[232]

There are thus indications that BCG not only provides non-specific protection against a broad range of diseases unrelated to TB. BCG also seems to make the immune system more effective by fortifying subsequent immune responses against diseases prophylactically targeted by routine vaccinations.

8.4 Paper I

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8.5 Paper II

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8.6 Paper III

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8.9 Study Protocol: Evaluating the effectiveness of different BCG strains in Guinea-Bissau: A randomized trial of the impact on neonatal hospital admissions.

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8.10 Study Protocol: A randomized trial of providing BCG vaccination immediately to neonates admitted to the intensive care unit in Guinea-Bissau: Effect on mortality

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8.11 Rear page summary

This thesis examines the non-specific effects associated with vaccination at birth with different BCG strains. We assessed the overall effects on hospital admission risk and in-hospital case-fatality of providing early BCG-Denmark versus delayed BCG, and of providing different strains of BCG.

Neither early BCG nor the different strains of BCG affected the risk of hospitalization, but BCG-Denmark versus no-BCG was associated with a substantial 42% reduction in in-hospital deaths, primarily due to a 54% reduction in cases of fatal neonatal sepsis.

It is documented in a large-scale RCT that different strains of BCG have different immunogenic profiles; BCG-Denmark and BCG-Japan thus induces more 2-month BCG skin reactions that are also larger, more 6-month TST conversions and more adverse events when compared to BCG-Russia. Across five trials providing BCG-at-birth, infants presenting a 2-month BCG skin reaction had a 50% lower subsequent all-cause mortality up to one year of age than infants presenting no 2-month reaction, and larger reaction sizes were associated with the greatest mortality reduction. In a meta-analysis of data regarding BCG-vaccinated infants from Guinea-Bissau, having a TST reaction at 2- or 6-months of age was associated with 44% and 37% reduced all-cause mortality compared to having no reaction.