Bacillus Calmette-Guérin vaccination at birth:

Effects on early childhood infections, growth, and development.

Jesper Kjærgaard

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Tutors: Gorm Greisen, Lone Graff Stensballe

Official opponents: Klaus Müller, Henrik Christesen, and Federico Martinón-Torres.

Correspondence: Research Unit Women's and Children's Health, Department of Paediatrics and Adolescent Medicine, Rigshospitalet and Research Center for Vitamins and Vaccines, Statens Serum Institut.

E-mail: jesper.kjaergaard@regionh.dk

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THE THREE ORIGINAL PAPERS:

1. Kjærgaard J, Birk NM, Nissen TN, Thøstesen LM, Pihl GT, Benn CS, Jeppesen DL, Pryds O, Kofoed P-E, Aaby P, Greisen G, Stensballe LG. Non-specific effect of BCG vaccination at birth on early childhood infections. A randomized, clinical multicenter trial. Pediatric Research. DOI: 10.1038/pr.2016.142. Online first August 17, 2016.
2. Kjærgaard J, Stensballe LG, Birk NM, Nissen TN, Thøstesen LM, Pihl GT, Nielsen AV, Aaby P, Kofoed P-E, Pryds O, Greisen G. Bacillus Calmette-Guérin immunemodulation at birth: Effects on infant growth. A randomized clinical trial. Early Human Development 100 (2016) 49-54. DOI: 10.1016/j.earlhumdev.2016.05.015.
3. Kjærgaard J, Stensballe LG, Birk NM, Nissen TN, Foss KT, Thøstesen LM, Pihl GT, Andersen A, Kofoed P-E, Pryds O, Greisen G. Lack of a negative effect of BCG-vaccination on child psychomotor development: results from The Danish Calmette Study - a randomised clinical trial. PLoS ONE 11(4):e0154541. DOI: 10.1371/journal.pone.0154541. Online first April 28, 2016.

From here on, these are referred to as Paper 1, Paper 2, and Paper 3 in the thesis.

1. INTRODUCTION

Yearly, approximately 1.4 billion vaccines are being given to children below the age of 1 year globally. The current understanding of vaccines has primarily been revolving around their ability to provide specific protection against a target disease. But recent evidence from both clinical and immunological studies tell another story, in which vaccines can have effects on health that extend beyond the protection against the target disease – a concept that has been known as non-specific effects of vaccines.

This thesis is based on three papers from the Danish Calmette Study, which is a randomized, clinical trial designed to assess the non-specific effects of Bacillus Calmette-Guérin (BCG) vaccination at birth in children up to 15 months of age. The outcomes of the Danish Calmette Study cover the clinical non-specific effects of BCG on infectious and allergic diseases, child growth and psychomotor development, as well as changes in the immune system induced by BCG vaccination, and a qualitative and mixed method exploration of parental perspectives on BCG vaccination.

The Danish Calmette Study is, to our knowledge, the largest randomized pediatric trial that has been conducted in Denmark. The work has been done in a collaboration consisting of 30 – 40 people at times. Each of the five PhD-students that have been part of conducting the study have had the responsibility for the daily routines of the research teams at each of the three study sites, as well as for a certain part of the data collection and the reporting of the associated outcomes.

This thesis addresses three outcomes from the Danish Calmette Study; the non-specific effect of BCG on infections (reported in Paper 1), effects on child growth (reported in Paper 2), and effects on psychomotor development (reported in Paper 3). All outcomes reported in this thesis were pre-specified in analysis plans that were deposited with the data and safety monitoring board prior to un-blinding the data.

The term 'primary outcome' will only be used concerning the primary outcome of the Danish Calmette Study (all-cause hospitalizations). Other outcomes will be termed 'the main outcome' of the paper and 'secondary outcomes' of the papers in this thesis.

2. BACKGROUND

2.1 Bacillus Calmette-Guérin vaccine

The BCG vaccine was developed by Albert Calmette and Camille Guérin in the beginning of the 20th century to protect against tuberculosis. They used Mycobacterium bovis, the bacteria that causes tuberculosis in cows, and sub-cultured it on an agar based on potatoes and glycerinated ox bile to make it less virulent. It took 239 passings over the course of 13 years to attenuate the bacteria sufficiently for use in humans without causing disease. In 1921 they used what is now called Bacillus Calmette-Guérin (BCG) for vaccinating humans against tuberculosis for the first time.[1] Today, 100 years after its introduction, BCG is still the only vaccine against tuberculosis. It is one of the most widely used vaccines in the world with an annual 180 million doses needed globally according to the World Health Organization (WHO).

The vaccine consists of live, attenuated BCG. Different strains have evolved due to variations in the attenuation process between different laboratories.[2] It is administered as an intradermal injection on the upper arm at the point of insertion of the deltoid muscle. It typically results in a localized inflammatory reaction with a pustule that will last for 2 to 8 weeks and will result in a scar at the site of injection. Common side effects include regional lymph node enlargement (> 1/100 vaccinated) and rarer side effects include a localized abscess and suppurative lymphadenitis (<1/1000 vaccinated). Systemic infection with the live attenuated BCG has been observed in immunocompromised children.[3]
The protection against tuberculosis is variable (about 40% to 80%), being best against severe, disseminated infections in childhood, as opposed to a lower protection against pulmonary tuberculosis in adults.[4,5] The global burden of tuberculosis remains high with approximately 9 million cases a year and tuberculosis is still among the top ten causes of death worldwide.[6]

2.2 BCG in the Danish national vaccination program
BCG is not currently part of the Danish national vaccination program (Table 1).[7] It was used for the prevention of tuberculosis in Denmark until the beginning of the 1980s, at which time it was gradually withdrawn due to decreasing incidence of tuberculosis. At that time, it was given to children around the age of 5 to 7 years, thus the latest routinely vaccinated birth cohorts are from approximately 1975-77. In Denmark, BCG is currently only recommended for persons, especially children and adolescents, who plan to stay for an extended period of time in parts of the world where tuberculosis is common and where close contact with the local population is expected.[8]

Table 1: The current Danish national vaccination schedule from 0 to 4 years.

Age	Vaccination
3 months	Diphteria, tetanus, pertussis, polio, *H. influenzae* type b (Hib) + pneumococci.
5 months	Diphteria, tetanus, pertussis, polio, *H. influenzae* type b (Hib) + pneumococci.
12 months	Diphteria, tetanus, pertussis, polio, *H. influenzae* type b (Hib) + pneumococci.
15 months	Measles, mumps, and rubella.
4 years	Measles, mumps, and rubella.

2.3 Unexpected clinical effects of BCG
Shortly after the introduction of BCG vaccination in Sweden in the 1920s, regional physician Carl Näslund noticed that the mortality among BCG vaccinated children was significantly lower than among the children that were not BCG vaccinated (Figure 1[9]). This was unexpected because tuberculosis is a rare cause of death during childhood, so Näslund concluded that the decreased mortality must have been due to prevention of something else and coined the phenomenon 'non-specific immunity'.[10] The same pattern is also suggested in historical data from England where an unexplained decrease in non-tuberculous mortality was observed among children vaccinated with BCG.[11]
In the 1970s it was discovered that BCG could be used to treat bladder cancer[1] and intravesical BCG is now standard treatment for superficial stages of the disease.
More recently, and in line with Näslund's initial oberservation[10], clinical observational studies[12–15] and a randomized

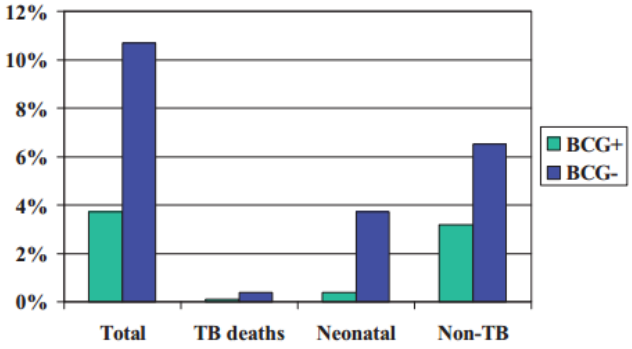

Figure 1: Introduction of BCG in Norrbotten, Sweden, 1927-31: Mortality at 0-4 years of age. 20 000 children. BCG: Bacillus Calmette Guerin. TB: Tuberculosis.

trial[16] from West Africa have shown decreases in mortality that could not be explained by the prevention of tuberculosis. Verbal autopsies showed that the effect was due to prevention of infections.[17] Other vaccines also seemed to have non-specific effects on mortality[18] and thus, the concept that vaccines can have health effects that extend beyond the protection against the target disease has now been coined non-specific effects of vaccines.[19]
The notion that BCG and other vaccines could have non-specific effects on mortality and hence the planning of vaccination programs, lead WHO to commission a review of non-specific effects of vaccines in 2012.[20] On the basis of that report, the WHO working group concluded that BCG could have non-specific effects but that more research should be conducted, preferably using randomized trial design in other settings than the low-income, high burden of disease, high mortality setting where most of the current evidence stems from, and that studies should be looking a broader range of outcomes than mortality.[21] This was the basis for the Danish Calmette Study, which this thesis is based on and which is described in detail in the methods section.

2.4 Immune response to BCG and basis for non-specific effects
BCG has been studied for several other purposes than prevention of tuberculosis, from preventing atopic eczema[22] to treating malignant melanoma[23], multiple sclerosis[24], and type 1 diabetes[25], as well as the established use for treating superficial bladder cancer.
The rationale for using BCG in these conditions is in the immune regulatory effects BCG exerts. The immune response to BCG is still not fully understood, but it generally involves a type 1 response[26] dominated by interferon-γ, interleukin (IL)-2, and tumor necrosis factor (TNF)-α[27], but also induces regulatory T-cells when immune cells of vaccinated individuals are exposed to mycobacterial antigens.[28]
Previously, no plausible biological mechanism for the non-specific prevention of infections by BCG seen in clinical studies was known, but recently several immunological mechanisms have been proposed to explain this. They include a recently recognized ability to induce training in the monocytes of the innate immune system ('trained immunity') via epigenetic programming[29], making the monocytes able to produce increased amounts of interferon-γ when exposed to unrelated pathogens up to one year after vaccination[30], and also induction of cross reactive T-cells ('heterologous immunity'), and increased antibody titers to subsequent vaccines.[31] The immune response to unrelated pathogens following BCG vaccination also involves increased levels of TNF-α, as well as IL-1β and IL-6.[29,32,33] These effects

provide some biological explanation of how BCG vaccination can have a preventive effect on unrelated infections.

2.5 Burden of childhood infections in Denmark

Childhood infections are the most common cause of illness in children in Denmark. Within the past 14 days, 19% of children below the age of one year have had a respiratory tract infection and 10% have been hospitalized for infection during the first year of life.[34] In 2010, parents in Denmark took a million days off from work to take care of their children when they were sick.[35] Thus, childhood infections represent a significant childhood health problem in Denmark.

2.6 Other possible effects of BCG

The immune system is central to healthy growth and neurodevelopment.[36,37] Given BCGs ability to induce a pronounced, complex, and long lasting immune responses as described above, BCG could also influence other important aspects of child health, such as child growth and psychomotor development.

2.6.1 BCG and child growth and body composition

Infections can impair child growth[38] and BCG could potentially reduce the incidence of childhood infections and thus increase child growth, but no clear effect of BCG on child growth was found in a low-income setting.[39]

Another possible mechanism for BCG to influence growth or body composition involves the inflammatory profile which BCG induces.[26] Inflammation is associated with insulin resistance and body composition.[40] When stimulated with unrelated antigens, the blood of BCG vaccinated children showed increased in vitro responses of the inflammatory cytokines TNF-alpha, IL-1beta, and IL-6 [33], which have been shown to induce insulin resistance.[41] A link between the intestinal microbiome and obesity has also been suggested[42] and a study has indicated that the change in microbiome preceded the occurrence of obesity.[43] Another study has shown a correlation between immune response to BCG vaccination and changes in intestinal microbiome, though it was not possible to determine the causality – BCG may have changed the microbiome or the microbiome may have altered the effect of BCG.[44] Thus there are several possible mechanisms by which BCG could affect child growth and body composition.

2.6.2 BCG and neonatal neuro-inflammation

Inflammation is part of the pathogenic cascade of perinatal brain damage, which can be triggered by e.g. a systemic infection, and can have a sensitizing effect to successive insults to the neonatal brain. Neonatal brain damage is more frequent among premature children and it is believed that the brains of premature children are more susceptible to damage than those of children born at term.[45] Some studies indicate that the detrimental effects of inflammation are more pronounced if the inflammatory stimulus is prolonged or intermittent.[46] As previously described, BCG induces inflammatory responses[47] and causes a localized inflammatory reaction for months.[3]

Some of the detrimental neonatal neuro-inflammatory responses are driven by Toll-like receptor 2 activation[46,48] which is one of the receptors used by innate immune cells to recognize BCG[49], thus supplying a possible mechanism by which BCG could adversely affect neonatal brain development.

On the other hand, BCG vaccination has been shown to attenuate the neuro-inflammatory effect of a subsequent neuro-insult in animal studies[50], perhaps by inducing regulatory T-cells[28], which have been associated with attenuation of neuro-inflammation[51], or by leaving microglia remaining in a resting state.[52] In a mouse model, healthy mice injected with BCG had improved behavioural performances and neurogenesis at 4 weeks.[53] Finally, the gut microbiome is important in neurodevelopment[54,55], and as described above, there is a possible link between BCG and the microbiome.[44]

Thus BCG can potentially have both beneficial and detrimental effects on child development and may have a more pronounced effect in premature children.

2.7 Summary of background

BCG vaccination has been shown in clinical studies in low-income, high burden of disease areas to have a beneficial effect on mortality by preventing infections. Several possible immunological mechanisms for this effect have recently been proposed. BCG vaccination has complex and long lasting effects on the immune system and may thus affect not only susceptibility to infections but also child growth and neuro-development.

The possible mechanisms by which BCG can affect childhood infections, growth, and psychomotor development are summarized in Figure 2.

3. OBJECTIVES OF THE THESIS

3.1 Hypotheses of the thesis

Studying an intervention that has a complex and long lasting effect on the immune system at birth calls for a broad view on possible effects. Especially when the intervention is applied in a different setting from where most of the previous evidence was generated. Thus, a holistic view on child health has been adopted in this thesis by acknowledging that biological interactions are complex and we, as clinical researchers, need to be as certain as possible that we do no harm with our interventions.

This thesis tries to assess whether BCG has effects on overall child health by assessing BCGs non-specific infection-preventing effects, as well as assessing growth and psychomotor development as generic indicators of child health.

It was hypothesized that BCG would lower the number of infections via non-specific effects on the immune system, that BCG could affect growth either via prevention of infections or perhaps via interaction with metabolic pathways, and that BCG could affect neurodevelopment perhaps through inflammatory mechanisms or via regulation of CNS immune responses.

3.2 Aims of the thesis

The aim of this thesis is:

1. To determine whether BCG vaccination at birth can decrease the number of infections (Paper 1).
2. To explore whether BCG vaccination has any beneficial or detrimental effects on child growth (Paper 2).
3. To explore whether BCG vaccination has any beneficial or detrimental effects on child development, with special attention to premature children (Paper 3).

4. MATERIAL AND METHODS

4.1 Overall design and participants

The Danish Calmette Study was a randomized, multicenter clinical trial, which allocated newborns to receive BCG or to a standard care control group from October 6th 2012 to November 29th 2013 at three maternity wards in Denmark.

All women who planned to give birth at the three study sites received a letter during the 2nd or 3rd trimester with an invitation to participate in the Danish Calmette Study. The recruitment

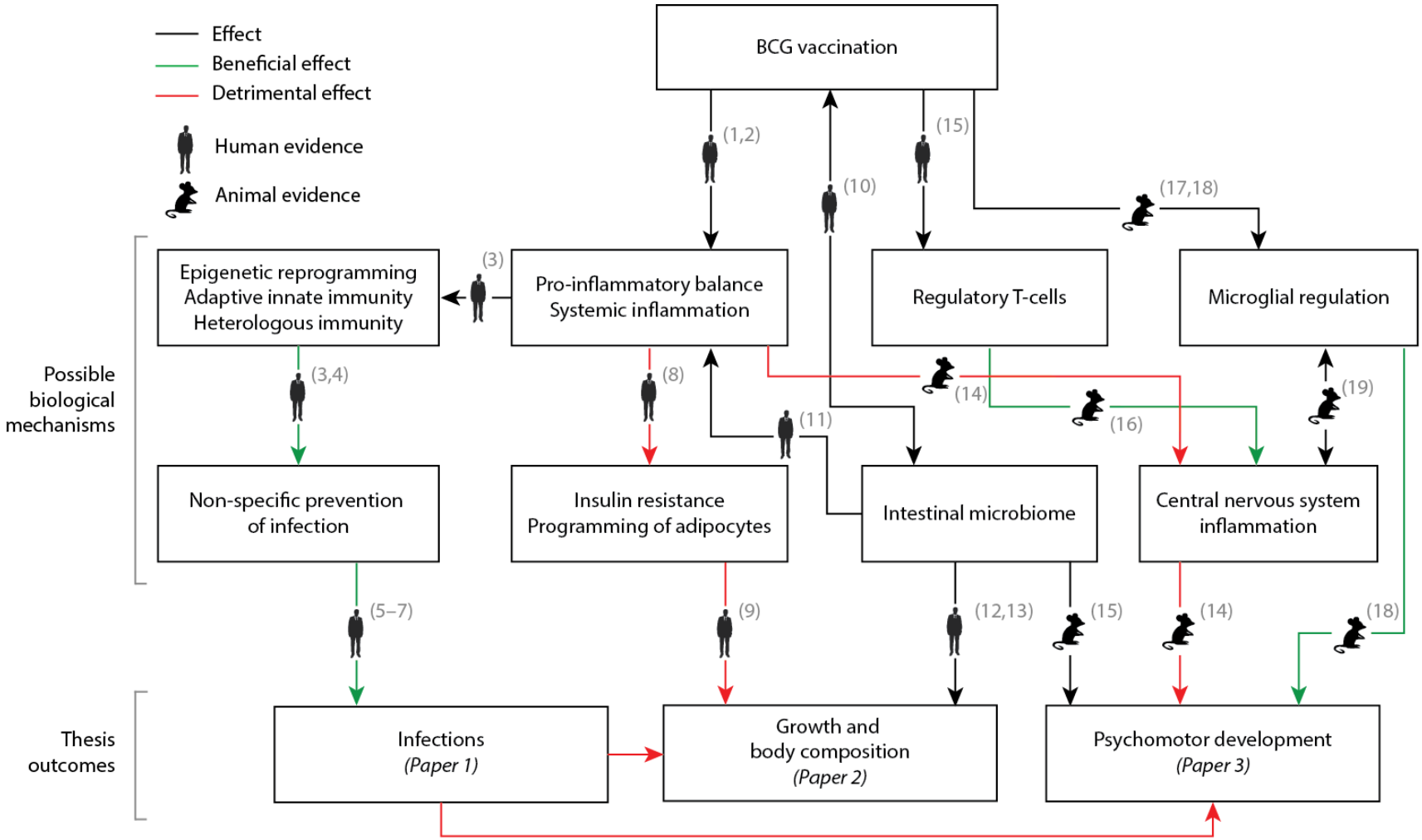

Figure 2: Possible mechanisms by which BCG can affect childhood infections, growth, and psychomotor development.

1. Ota MO, Vekemans J, Schlegel-Haueter SE, et al. Influence of Mycobacterium bovis bacillus Calmette-Guérin on antibody and cytokine responses to human neonatal vaccination. *J Immunol*. 2002;168(2):919-925; 2. Marchant A, Goetghebuer T, Ota MO, et al. Newborns develop a Th1-type immune response to Mycobacterium bovis bacillus Calmette-Guérin vaccination. *J Immunol*. 1999;163(4):2249-2255; 3. Kleinnijenhuis J, Quintin J, Preijers F, et al. Bacille Calmette-Guerin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. *Proc Natl Acad Sci USA*. 2012;109(43):17537-17542; 4. Netea MG, Quintin J, van der Meer JWM. Trained immunity: a memory for innate host defense. *Cell Host Microbe*. 2011;9(5):355-361; 5. Aaby P, Roth A, Ravn H, et al. Randomized trial of BCG vaccination at birth to low-birth-weight children: beneficial nonspecific effects in the neonatal period? *J Infect Dis*. 2011;204(2):245-252; 6. Storgaard L, Rodrigues A, Martins C, et al. Development of BCG Scar and Subsequent Morbidity and Mortality in Rural Guinea-Bissau. *Clin Infect Dis*. 2015;civ452; 7. Stensballe LG, Nante E, Jensen IP, et al. Acute lower respiratory tract infections and respiratory syncytial virus in infants in Guinea-Bissau: a beneficial effect of BCG vaccination for girls community based case-control study. *Vaccine*. 2005;23(10):1251-1257; 8. Chen L, Chen R, Wang H, Liang F. Mechanisms Linking Inflammation to Insulin Resistance. *Int J Endocrinol*. 2015;2015:508409; 9. Weiss R, Dziura J, Burgert TS, et al. Obesity and the metabolic syndrome in children and adolescents. *N Engl J Med*. 2004;350(23):2362-2374; 10. Huda MN, Lewis Z, Kalanetra KM, et al. Stool microbiota and vaccine responses of infants. *Pediatrics*. 2014;134(2):e362-e372; 11. Devaraj S, Hemarajata P, Versalovic J. The human gut microbiome and body metabolism: implications for obesity and diabetes. *Clin Chem*. 2013;59(4):617-628; 12. Cryan JF, Dinan TG. Mind-altering microorganisms: the impact of the gut microbiota on brain and behaviour. *Nat Rev Neurosci*. 2012;13(10):701-712; 13. Borre YE, O'Keeffe GW, Clarke G, Stanton C, Dinan TG, Cryan JF. Microbiota and neurodevelopmental windows: implications for brain disorders. *Trends Mol Med*. 2014;20(9):509-518; 14. Hagberg H, Mallard C, Ferriero DM, et al. The role of inflammation in perinatal brain injury. *Nat Rev Neurol*. 2015;11(4):192-208; 15. Boer MC, Joosten SA, Ottenhoff THM. Regulatory T-Cells at the Interface between Human Host and Pathogens in Infectious Diseases and Vaccination. *Front Immunol*. 2015;6(May):1-15; 16. González H, Pacheco R. T-cell-mediated regulation of neuroinflammation involved in neurodegenerative diseases. *J Neuroinflammation*. 2014;11:1-11; 17. Yong J, Lacan G, Dang H, et al. BCG vaccine-induced neuroprotection in a mouse model of Parkinson's disease. *PLoS One*. 2011;6(1):e16610; 18. Yang J, Qi F, Gu H, et al. Neonatal BCG vaccination of mice improves neurogenesis and behavior in early life. *Brain Res Bull*. 2016;120:25-33. 19. Czeh M, Gressens P, Kaindl AM. The yin and yang of microglia. *Dev Neurosci*. 2011;33(3-4):199-209.

period was from September 2012 to November 2013. Study staff called the families on the telephone to answer questions and to conduct a recruitment interview with families willing to participate. Exclusion criteria were: Gestational age < 32 weeks, birth weight < 1000 g, critically ill newborn, known or suspected immune deficiency, maternal use of immune suppressing medicine during pregnancy, or, because we used questionnaires in Danish, no Danish-speaking parent. Exclusion criteria were reviewed after birth, prior to randomization.

4.2 Intervention

The intervention was BCG vaccination (SSI strain 1331) in the standard pediatric dose of 0.05 mL within seven days of birth. The vaccine was given intradermally at the point of the insertion of the deltoid muscle on the left side. Children in the control group received standard care.

4.3 Thesis outcomes

This thesis addresses three predefined outcomes from the Danish Calmette Study:

(1) Parent reported infections.

(2) Growth and body composition.

(3) Psychomotor development.

Initially, we had planned to study child development only as a safety measure in premature children, who are thought to be more susceptible to the inflammatory effects of BCG, but recruitment of premature children turned out to be challenging, and, following ethical approval, we extended the data collection on child development to the entire population at age 12 months to gain more power to ascertain the safety of BCG concerning child development (Paper 3).

4.4 Data collection

Baseline data were collected by an initial telephone interview prior to randomization. Previous research has indicated that the non-specific effects of BCG can be altered by diphtheria-tetanus-pertussis vaccination.[56] For this reason, data on outcomes were collected at age 3 and 13 months, as this would result in a period from birth to 3 months, where BCG was the only vaccine given and a period from 3 to 13 months in which Danish children receive diphtheria-tetanus-pertussis-polio and *H. influenzae* type b and pneumococci vaccinations (Table 1). The outcome data were collected using telephone interviews, clinical examinations, and parent reported questionnaires.

4.4.1 Infections

Data on child infections were collected at the telephone interviews using a structured questionnaire. The parents were asked about episodes of common cold, physician diagnosed pneumonia, fever with no apparent cause, diarrhea and vomiting, and acute otitis media, as well as number of visits to the general practitioner due to suspected infection. Parents were given an infectious disease diary at birth and asked to take notes of their child's illness. At the beginning of the telephone interviews, parents were asked not to reveal the randomization group of the child in order to keep study staff blinded during data collection (Paper 1).

4.4.2 Growth and body composition

Data on child growth and body composition were collected at the clinical examinations. At 3 months, weight and length was recorded, and at 13 months additionally, mid upper-arm circumference (MUAC) and subscapular and triceps skinfold thickness was measured to be able to address possible changes in body composition. All measurements were recorded in triplicate and averaged, except weight which was only measured once per child. The anthropometric data were standardized to z-scores using WHOs reference population.[57,58] In order to keep study staff blinded to randomization group of the child during data collection, parents covered the sore/scar if in the BCG group, or the typical injection site if in the control group, with a plaster prior to clinical examination (Paper 2).

4.4.3 Psychomotor development

Data on child psychomotor development were collected using the parent reported Ages and Stages Questionnaire, 2nd edition (ASQ).[59] The questionnaires were adapted for online use and distributed to the families at child age 12 months. The parents of premature children born at GA < 37 weeks additionally received a questionnaire at 6 and 22 months of age. Due to a programming error, premature children below GA 33 weeks and 4 days were not flagged by our system and thus they did not receive the 6 months ASQ.

The ASQ contains 30 items within 5 subdomains on everyday activities performed by children.[59] The questionnaire was available to the parents for five days after they initially started completing it, in order to ensure that the score reflected the current developmental stage of the child. Each question could be answered with a 'yes', 'sometimes', or 'not yet', scoring 10, 5, or 0 points. The score was added up into a total ASQ score, ranging from 0 to 300.

The ASQ scores were reported in both actual scores and standardized into Cohen's d, which is essentially the difference between two means measured in standard deviations. Thus Cohen's d supplies a way of evaluating the size of a difference on an arbitrary or not easily interpreted scale (Paper 3).

Figure 2 provides an overview of the data collection process.

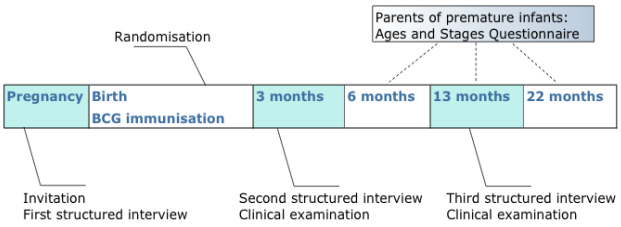

Figure 2: Overview of the data collection process

4.5 Sample size and power

The sample size was determined by the primary outcome of the Danish Calmette Study: All-cause hospitalizations until age 15 months. To detect a 20% reduction in all-cause hospitalization by 15 months of age with 90% power and 0.05 as significance level, 3972 children were needed. We aimed to include 4300 children. The detectable effect sizes of the outcomes reported in this thesis were:

1. A 22% reduction in parent reported infectious illnesses at 13 months with a power of 94%. (Paper 1)
2. A difference in weight of 0.1 standard deviations (SD) at 13 months, equivalent to approximately 100 g, with a power of 90%. (Paper 2)
3. A difference in mean ASQ score of effect size 0.13 at one year, equivalent to approximately 5 ASQ points, with a power of 98%. (Paper 3)

4.6 Randomization and blinding

Newborn children were randomized 1:1 by the midwife or by study staff. Randomization was conducted within 7 days of birth, using a centralized on-line system with stratification according to GA of the child (< 37 weeks vs. ≥ 37 weeks). The allocation sequence was computer generated in permuting blocks of 2:4:6. Study staff was blind to randomization at data collection, but it was not feasible to blind the parents (or the child) to the intervention due to the pustule and scar that follows BCG vaccination (Figure 4).

4.7 Statistics

4.7.1 Subgroup analyses

Subgroup analyses of child sex and prematurity were pre-planned for the Danish Calmette Study because previous studies have suggested differential effects of BCG in these groups.[16,60] A sub-group analysis of maternal BCG vaccination status was also pre-specified due to a previous study concluding that non-specific effects of the measles vaccine are more pronounced when given in the presence of maternal antibodies.[61]

4.7.2 Interim analysis

The trial was to be terminated if BCG had a negative effect ($p < 0.01$) or a positive effect ($p < 0.001$) on GP visits and/or on parental report of eczema, as obtained through the telephone interviews. The data and safety monitoring board conducted interim analyses in February 2013, August 2013, and February 2014.

4.7.3 Statistical analyses

Categorical data were analyzed using $\chi 2$-test (Paper 1). Continuous non-normally distributed data were analyzed using Mann-Whitney U-test (Paper 1 and 3). Count data were analyzed using negative binomial regression models (Paper 1). Continuous, normally distributed outcomes (anthropometric data and ASQ scores) were analyzed using general linear models (Paper 2) and general linear models adjusted for age at questionnaire completion (Paper 3).

All statistical analyses used a two-tailed significance level of 0.05. For papers in this thesis, all analyses were adjusted for prematurity in accordance with the stratification of the randomization and were conducted as intention to treat analyses. Per-protocol analyses were conducted for sensitivity. Secondary outcomes were subject to Holland correction[62] for multiple testing. All statistical analyses of the outcomes in the papers were described in the statistical analysis plan, except if otherwise stated. The statistical analysis plan was finalized and deposited with the data and safety monitoring board prior to un-blinding the data.

One post-hoc analysis (Mixed Model Repeated Measurements) was performed, pooling the three data points (ASQ score at 6, 12, and 22 months) for the premature children (Paper 3).

An overview of the methods in each paper is provided in Table 2.

4.8 Informed consent procedure and ethics approval

Informed consent was obtained on the telephone prior to birth from the majority of participants and reconfirmed immediately prior to randomization or, for a minority of participants, was obtained in person, after birth with sufficient time for considering allowed.

The Danish Calmette Study was approved by the Committees on Biomedical Research Ethics (J.no. H-3-2010-087), the Danish Data

Table 2: Overview of methods in Paper 1, 2, and 3.

	Paper 1	Paper 2	Paper 3
Follow-up	3 and 13 months	3 and 13 months	6, 12, and 22 months
Data	Telephone interview	Clinical examination	Parent reported online questionnaire
Outcomes	No. of infections	Growth and body composition	Psychomotor development
Subgroups	Child sex, prematurity, maternal BCG vaccination	Child sex, prematurity, maternal BCG vaccination	Child sex, prematurity, maternal BCG vaccination
Statistical analysis	Negative binomial regression	General linear models	General linear models

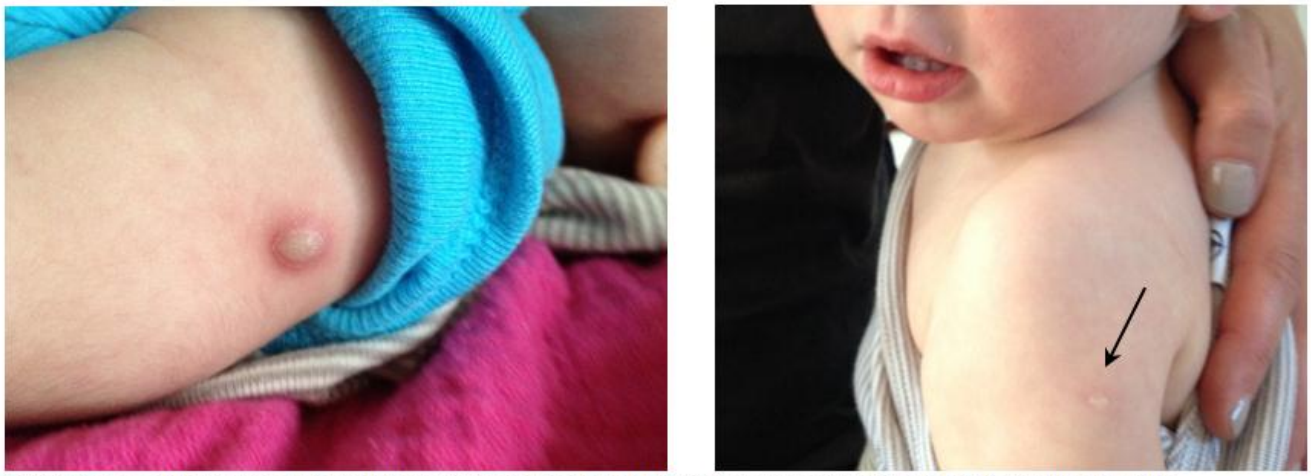

Figure 4: Left: Normal reaction with pustule at the site of BCG injection approximately 12 weeks after the vaccination. Right: Normal scar approximately one year after vaccination.

Protection Board (J.no. 2009-41-4141), and the Danish Medicines Agency (J.no. 2612-4356. EudraCT 2010-021979-85. Protocol 2009-323) and was registered at www.clinicaltrials.gov with trial registration number NCT01694108 prior to commencement. The trial was conducted under the supervision by the Good Clinical Practice Units of the Capital Region and the Region of Southern Denmark. The study was monitored by an independent data and safety monitoring board.

5. SUMMARY OF RESULTS

5.1 Enrollment and baseline characteristics

During the 13 months recruitment took place, 16 521 families were invited to participate. We randomized 4262 children. An overview of the participant flow can be found in Figure 3. The intervention and control group was balanced on the measured possible confounders, except for non-Danish ethnicity and paternal smoking during pregnancy, which were more frequent in the control group (Table 3).(Stensballe et al. 2016a)

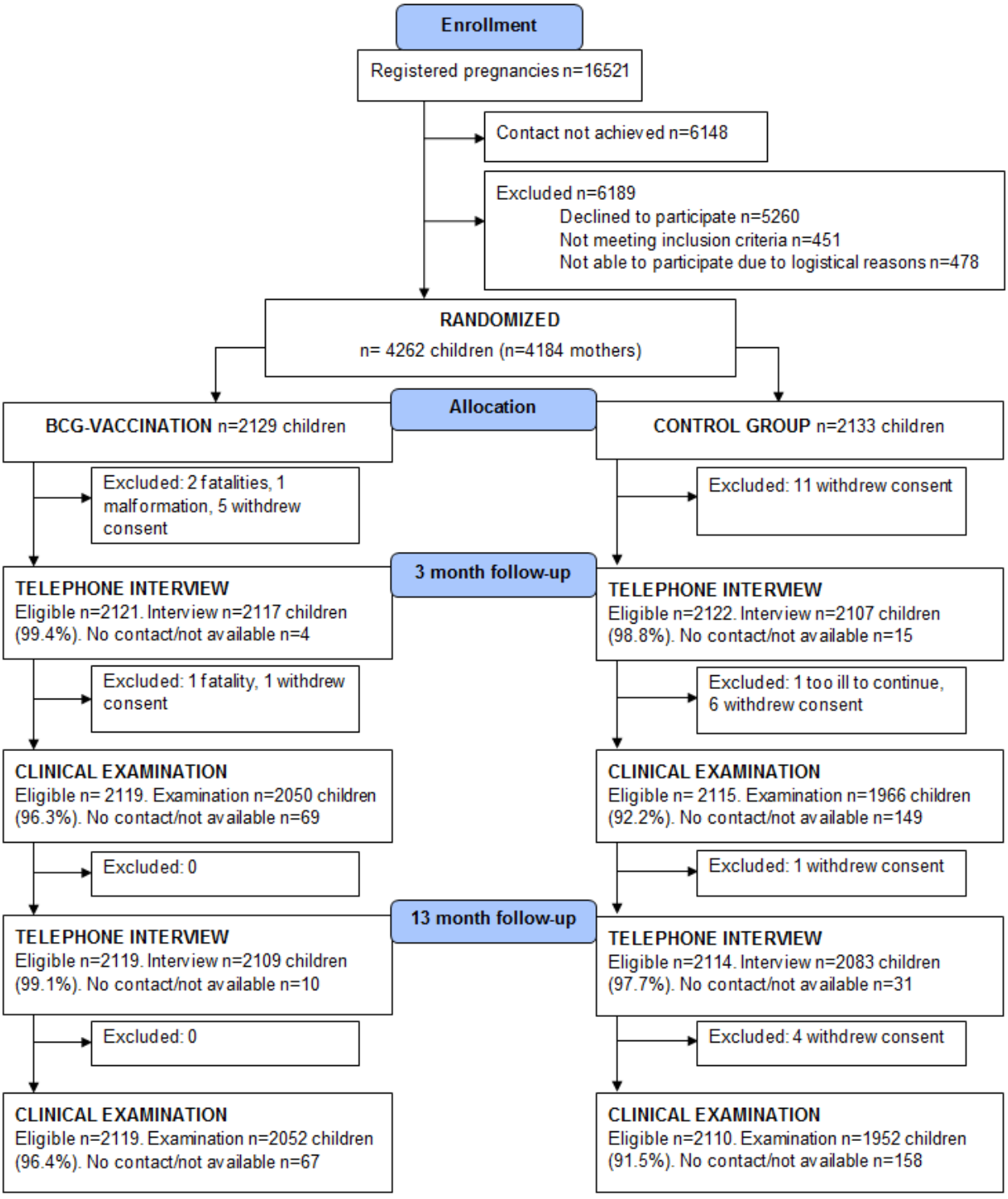

Figure 3: Flowchart of participants in the Danish Calmette Study

Table 3: Baseline characteristics by allocation group among 4184 mothers.

	BCG (n = 2095) n (%[a]) [NA]	Control (n = 2089) n (%[a]) [NA]
Boys[b]	1092 (52.1) [0]	1104 (52.9) [0]
Prematurity (GA < 37 weeks)	61 (2.9) [0]	60 (2.9) [0]
Randomization site	[0]	[0]
Rigshospitalet	751 (35.9)	780 (37.3)
Hvidovre	730 (34.8)	719 (34.4)
Kolding	614 (29.3)	590 (28.4)
Caesarean section	410 (19.6) [0]	442 (21.2) [0]
Antibiotics during delivery	333 (15.9) [0]	358 (17.1) [0]
Singletons	2061 (98.4)	2046 (97.9)
Twins	[0]	[0]
Triplets	34 (1.6)	42 (2.0)
	0	1 (0.0)
Birth weight in grams (mean ± SD)	3519 ± 493 [0]	3523 ± 494 [0]
Child < 1 day of age at randomization	1006 (48.0) [0]	1000 (47.9) [0]
Maternal age in years at birth (mean ± SD)	32.0 ± 4.6 [0]	31.9 ± 4.4 [0]
≥ 1 parent of other ethnicity than Danish	376 (18.1) [14]	458 (22.1) [15]
Maternal education	[7]	[6]
No higher education	460 (22.0)	435 (20.9)
Short/medium higher education	935 (44.8)	939 (45.1)
Long higher education	693 (33.2)	709 (34.0)
Parents living together	1984 (94.9) [4]	1984 (95.0) [0]
Mother BCG-vaccinated	364 (17.6) [27]	353 (17.2) [41]
≥ 1 older sibling	887 (42.4) [1]	843 (40.4) [2]
Atopic disposition[c]		
Maternal atopic disease	839 (41.6) [80]	809 (40.3) [83]
Paternal atopic disease	717 (38.2) [217]	708 (37.7) [209]
Parental atopic disease	1265 (65.7) [168]	1243 (64.1) [151]
Siblings with atopic disease	275 (13.3) [30]	252 (12.2) [23]
Smoking during pregnancy		
Maternal	203 (9.7) [1]	212 (10.2) [1]
Paternal	388 (18.9) [38]	458 (22.2) [31]

[a] Percentage among families where the information was available. [b] The sex of the 1st child in multiple births. [c] Atopic disposition defined as physician diagnosed atopic eczema, asthma, allergic rhinoconjunctivitis or food allergy. Abbreviations: BCG: Bacillus Calmette-Guérin vaccine. GA: Gestational age. NA: Not available (number of families without information on the specified variable). SD: Standard deviation.

5.2 Parent reported infections (Paper 1)

Follow-up was 99% and 98% complete at 3 and 13 months (Figure 3). There was no overall effect of BCG from 0 to 3 months or from 3 to 13 months of age, incidence rate ratio (IRR) = 0.87 (0.72 to 1.05) and IRR = 1.02 (0.97 to 1.07) respectively (Table 4). In the subgroup analysis of maternal BCG vaccination, there was an IRR of 0.62 (0.39 to 0.98) for infections from 0 to 3 months, corresponding to a number needed to vaccinate of 14. There were no statistically significant effects in subgroup analysis of child sex or prematurity (Figure 6).

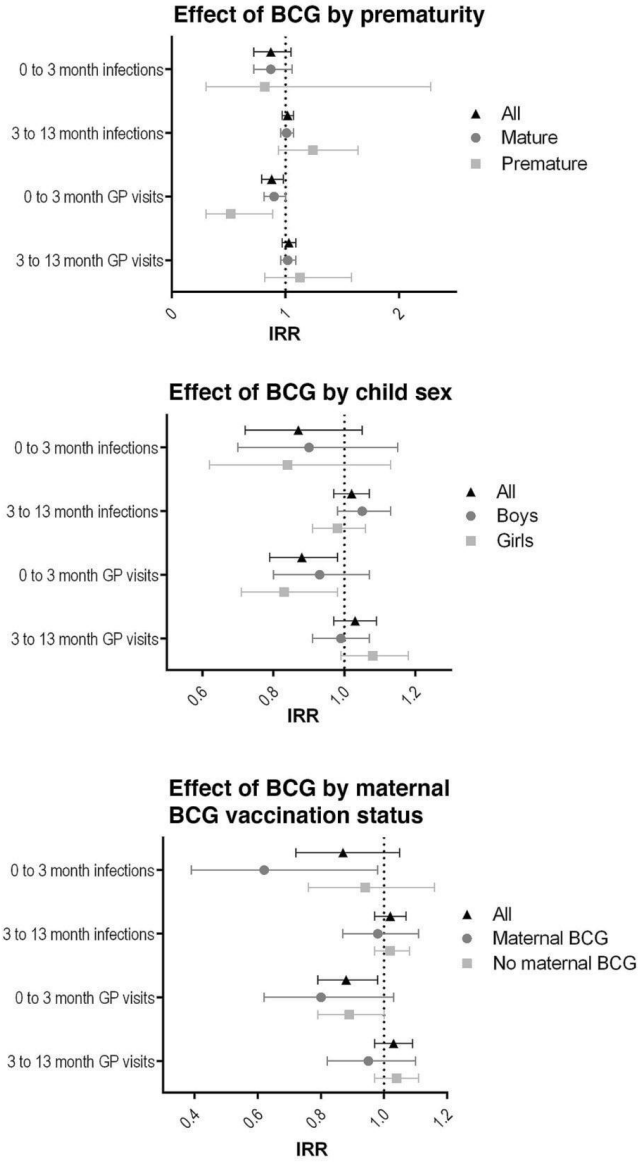

Figure 6: Subgroup analyses of BCGs effect on infections. BCG: Bacillus Calmette-Guérin. GP: General practitioner. IRR: Incidence rate ratio.

Table 4: Parent reported infections at 3 and 13 months (Paper 1)

Episodes of infectious illness[a]	BCG group (n = 2129)		Control group (n = 2133)		IRR[b] (95% CI), BCG vs. control
	n	Rate (events/months)	n	Rate (events/months)	
Birth to 3 months	2113	0.05 (291/5580)	2099	0.06 (336/5571)	0.87 (0.72 to 1.05)
Three to 13 months	2104	0.34 (7028/20526)	2072	0.33 (6791/20276)	1.02 (0.97 to 1.07)

[a] Parent-reported episodes of cold, pneumonia, fever, otitis media and diarrhea. [b] Incidence rate ratio; negative binomial regression adjusted for prematurity with months at risk as exposure. BCG: Bacillus Calmette-Guérin vaccine. IRR: Incidence rate ratio. 95% CI: 95% confidence interval.

5.3 Growth and body composition (Paper 2)

Follow-up was 94% complete at both 3 and 13 months (Figure 3). BCG did not have any effect on weight z-score at 13 months, BCG vs. control: -0.028 z-scores (-0.085 to 0.029), p = 0.34. There was no effect on weight or length at 3 months, or length, MUAC, or triceps and subscapular skinfold at 13 months, or in subgroup analyses of child sex, prematurity, or maternal BCG vaccination status (Figure 5).

5.4 Child psychomotor development (Paper 3)

Follow-up was approximately 80% complete, except for premature children at 6 months where it was only 68% (Figure 6), partly due to a technical error. The effect of BCG on ASQ score at 12 months was -0.7 points (-3.7 to 2.4), p = 0.67, corresponding to an effect size of Cohen's d = -0.015 (-0.082 to 0.052). The mean difference in ASQ score for premature children at 22 months was -7.8 points (-20.6 to 5.0, p = 0.23), d = -0.23 (-0.62 to 0.15). The effect remained negative but statistically insignificant when analyzing the overall BCG effect at 6, 12, and 22 months in the premature sub-group. There was no effect of BCG in subgroup analysis of child sex or maternal BCG vaccination status. The results are summarized in Figure 4.

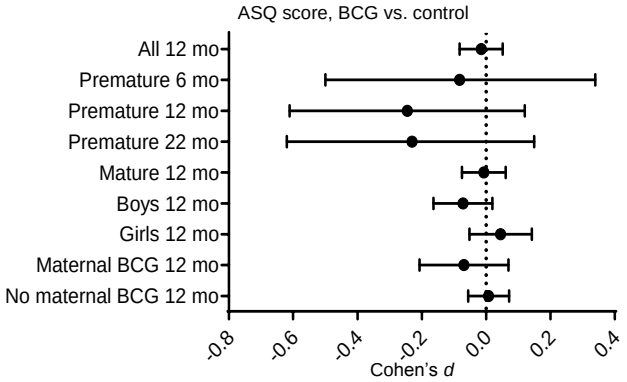

Figure 4: Effect of BCG on child development in subgroups. ASQ: Ages and stages questionnaire. BCG: Bacillus Calmette Guérin. Mo: months.

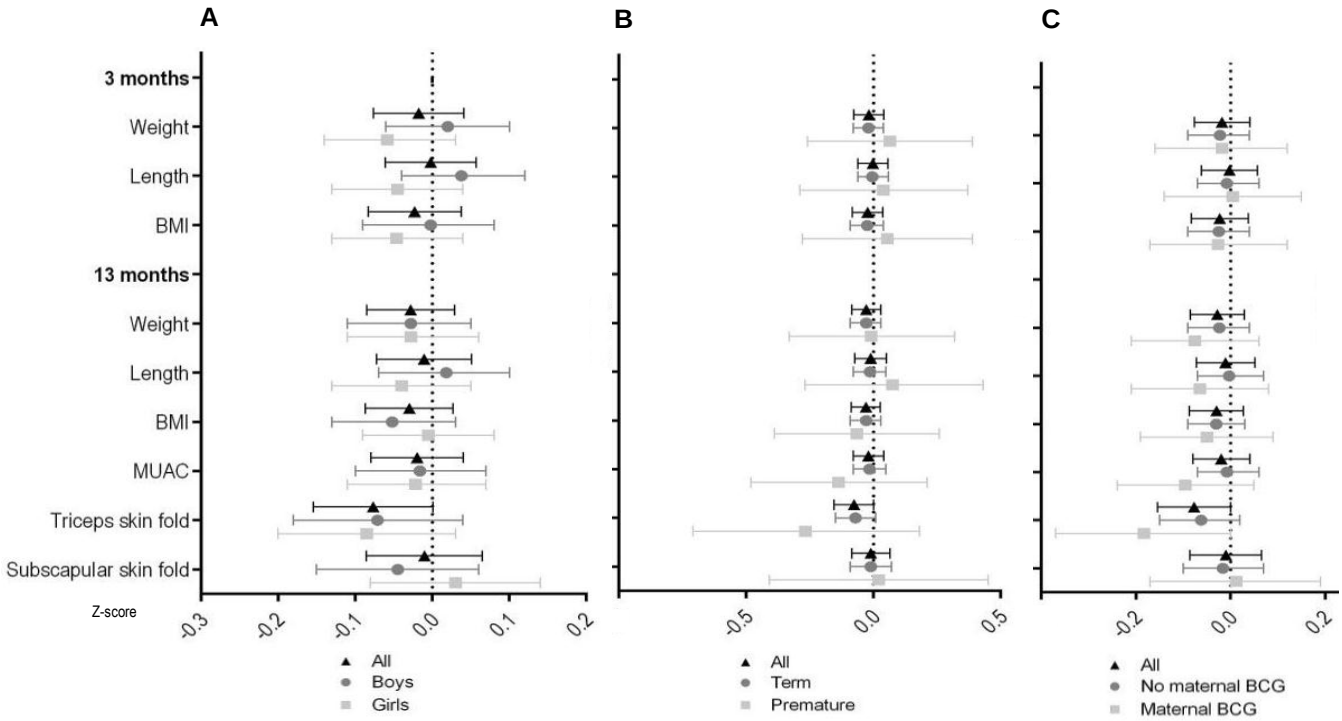

Figure 5: Subgroup analyses of BCGs effect on child growth and body composition. A: Effect of BCG by child sex, B: Effect of BCG by prematurity, C: Effect of BCG by maternal BCG vaccination status. BCG: Bacillus Calmette-Guérin vaccine. BMI: Body mass index. MUAC: Mid upper-arm circumference.

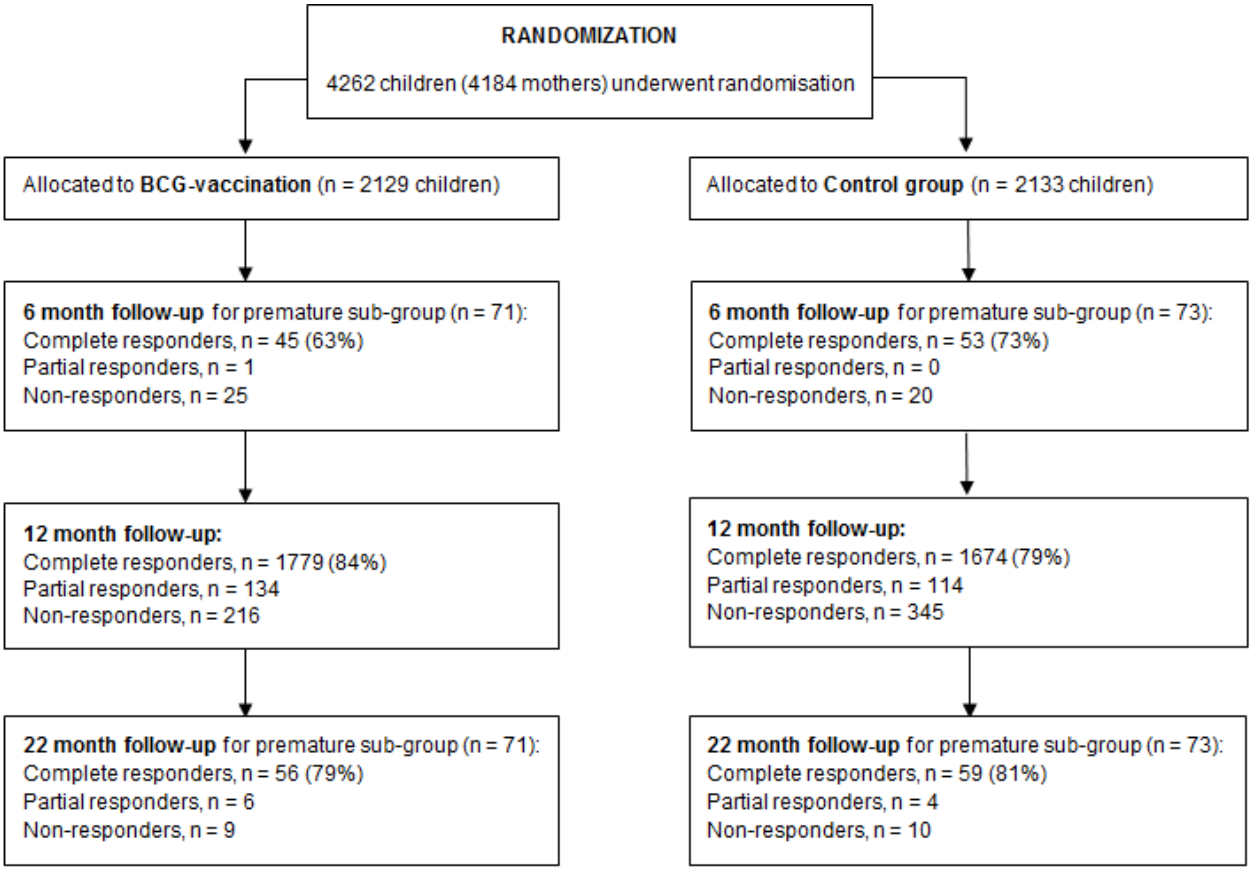

Figure 6: Flowchart of follow-up in Paper 3.

5.5 Conclusions (Paper 1, 2, and 3)

There was no overall, clinically relevant effect of BCG on infections from birth to 13 months, but BCG may have prevented 38% of infections from 0 to 3 months if the mother was BCG vaccinated (Paper 1). BCG vaccination did not affect child growth or body composition until 13 months of age (Paper 2). BCG did not have any effect on the development of children, but a non-significant negative point estimate was observed in premature children until age 22 months (Paper 3).

6. DISCUSSION

The papers in this thesis have addressed whether BCG vaccination at birth affects child health within the first year of life in a high income population. This was done by studying the impact of BCG vaccination on several generic child health outcomes, some of which BCG could possibly have both beneficial and detrimental effects on. BCG vaccination did not have any overall benefit on the prevention of infections, nor did it have beneficial or detrimental clinically relevant effects on growth or development, though our study did not have power enough to exclude a relevant negative effect on the development of premature children.

6.1 Consistency with previous findings

The lack of effect on prevention of infections was unexpected in the light of the previous research on non-specific prevention of infections.[12–16] A possible explanation could be that the immune response to BCG differs according to geographical location and setting[63], as well as exposure to other mycobacteria[64], all of which differs in the Danish Calmette Study from where most of the previous research has been done. Also the range and severity of infections are different between high- and low-income settings. Another possibility is that a type 2 error has occurred: Our study had a power of approximately 94% to detect an approximately 22% reduction in parent reported infections (Paper 1), and the results presented in this thesis could be the one in twenty accepting a false null hypothesis, or an actual effect could be smaller than 22%. Yet another possible explanation for this concerns the subgroup of BCG vaccinated mothers, which is discussed in the section on subgroup analyses below.

The literature on BCGs effect on growth and psychomotor development is sparse. BCGs effect on child growth has been studied in a low-income setting[39] and in older studies.[65,66] Previously, BCGs effects on psychomotor development has only been directly studied in a mouse model.[53] Thus, the results concerning growth and development are exploratory in their nature, but nonetheless, overall reassuring and indicate that the pro-inflammatory profile that BCG induces in the immune system does not affect growth, body composition, or development in a clinically relevant manner.

6.1.1 Subgroup analyses

A pre-planned subgroup analysis showed a protecting effect of BCG vaccination on infections from birth to 3 months (IRR = 0.62, CI: 0.39 to 0.98), if the mother had received BCG herself (Paper 1). Most of the previous research has been conducted in low-income settings[18,20] where maternal exposure to BCG or environmental mycobacteria is more frequent, or in high-income settings

where BCG vaccination is part of the public vaccination program.[67] This provides a possible explanation for the lack of overall effect on prevention of infections in the Danish Calmette Study, where there was no effect on either all-cause hospitalizations (Stensballe et al. 2016a) or parent reported infections (Paper 1). There was an effect on hospitalizations for infections if the mother was BCG vaccinated, and, in accordance with the findings from Paper 1, the effect was strongest during the first three months.(Stensballe et al. 2016b)

If BCG has a true preventive effect of a relevant size on infections among children of BCG vaccinated mothers, then there are at least two possible explanations for why the effect was most pronounced the first three months. First, previous research has shown that the non-specific effects of vaccines can be modulated by subsequent vaccines, and in our study, the first three months was a period in which no other vaccines had been given, thus supplying a clean effect of BCG. The effect may have disappeared due to the subsequent diphtheria-tetanus-acellular-pertussis-polio H. influenza type b + pneumococcus vaccination.[56] Second, the immune response needed for the non-specific prevention of infections may wane as the live attenuated bacteria are gradually cleared from the pustule during the course of a few months. But, this possibility is contradicted by evidence of a training effect of BCG on monocytes and on heterologous immunity that was still present 12 months after BCG vaccination in adults.[30]

Even if BCG vaccination at birth prevents infections in the child if the mother is BCG vaccinated, there was no effect of a relevant size on either growth or development in this subgroup, indicating that either the infections prevented did not affect growth or development at follow-up, or that BCG does not affect these three outcomes via the same mechanisms (Figure 2), or that the impact of BCG on growth and development was not yet manifest at our follow-up.

Looking at the premature subgroup, there was a significantly increased risk of hospitalization for infection (HR = 1.97, CI: 1.01 to 3.73) (Stensballe et al. 2016b) and a tendency towards a higher incidence of parent reported infections from 3 to 13 months (IRR = 1.24, CI: 0.94 to 1.64) (Paper 1), which taken together perhaps indicates that BCG vaccination of premature neonates in a high-income setting may have a detrimental non-specific effect on infections. These results were perhaps even more unexpected than the overall lack of effect on infection prevention, because previous research indicated that low-birth weight children has more pronounced non-specific effects of BCG on mortality.[16] If corroborated, the increased risk of infections among BCG vaccinated premature children, along with a non-significant negative tendency for BCGs effect on development (Paper 3), suggests that BCG vaccination of premature newborns should only be done on firm indications in high-income settings, i.e. expected exposure to tuberculosis from birth. Conversely, in low-income settings, where both the current evidence on non-specific effects of BCG in low birth weight children[16] and WHOs guidelines[68] suggests that BCG vaccination should be given as soon as possible after birth, regardless of prematurity.

The papers in this thesis present no evidence of sex-differential effects of BCG vaccination on infections, growth, or psychomotor development.

6.1.2 No effect vs. no effect of a clinically relevant size

When speaking of whether or not an intervention has an effect, there is an implicit judgement that the effect has to be of a size that is clinically relevant. It is quite possible that there is a biological effect of an intervention, even if a clinical study finds no effect, because the clinical outcome measure is not sensitive enough to change due to the biological effect, or that the change induced is not big enough to be discovered. These small effects could be teased out with a sample size that is big enough, though it would not serve a clinical purpose if the effect was so small that it would have no clinical impact for the patients for whom the intervention is targeted. Thus the term "no effect" in randomized trials with a clinical outcome often arguably equates "no effect of a relevant size" – this is the case for the papers in this thesis for example.

6.2 Methodological considerations

6.2.1 Strengths

There are several general strengths of The Danish Calmette Study. The randomized design, and blinding where it was feasible, helps decrease the risk of bias. Even though single blind studies tend to overestimate effects[69,70], blinding of parents was not feasible. We used broad inclusion criteria, resulting in a heterogeneous study population. We managed to recruit 10.2 families pr. day on average during the 410 days we included participants. We succeeded in achieving the target number of participants and had very good follow-up rates (Paper 1 and 2). For the main outcomes, we had sufficient strength to detect clinically relevant effects (Paper 1, 2, and 3). We explored previously unstudied outcomes, thus broadening the knowledge of the effects BCG (Paper 2 and 3). Only few participants did not adhere to their randomization group and either got BCG vaccinated when being allocated to the control group (n = 36) or did not receive BCG vaccination even though randomized to the intervention group (n = 11) (Figure 3). There were no differences between intention-to-treat and per protocol analyses (Paper 1, 2, and 3). Furthermore, the statistical analysis plan was deposited with the data and safety monitoring board prior to un-blinding the data.

Considering infectious disease episodes, the data collection used narrow definitions of diseases, thus increasing the likelihood that the parent reported infections correspond to an actual clinical infection (Paper 1).

The risk of performance bias on behalf of the un-blinded parents is low for anthropometric measurements, thus increasing the confidence in the findings (Paper 2).

We measured child development using a validated questionnaire and succeeded in obtaining a statistically optimal distribution of the ASQ scores, thus increasing the power of the study (Paper 3).

6.2.1.1 Choice of clinical outcomes vs. mechanistic biomarkers

Another approach to studying the effect of BCG vaccination at birth on child growth, body composition, and psychomotor development (Paper 2 and 3) could have been to use a much smaller sample, but evaluate it using a wider range of more detailed biological mechanistic outcomes in order to illuminate possible mechanisms of action (Figure 2), instead of addressing whether it has a clinical effect. But, if a wide range of surrogate outcomes had been chosen, it would carry an increased risk of type 1 errors (depending on the choice of multiple testing correction method) and could thus result in an array of interpretations and further studies being initiated on the grounds of random variation in surrogate biomarkers with uncertain clinical value.[71] On average, randomized controlled trials using surrogate markers as outcomes report treatment effects that are 48% higher, and report a beneficial effect twice as often as studies using clinical endpoints.[72]

Even if BCG vaccination has an actual effect on some of the biological mechanisms proposed in Figure 2, the finding of no clinically relevant effects of BCG vaccination on growth and psychomotor development supplies a concrete answer to a clinical question. Thus, the use of fairly crude, commonly used, clinically interpretable, overall measures of child health in a large sample could be seen as one of the major strengths of the papers in this thesis.

6.2.2 Limitations

There are some general limitations that apply to the Danish Calmette Study cohort. Only 1 in 4 invited families participated, but 40% of the families that were actually in contact with study staff were randomized (Figure 3). The cohort consisted of families where the mother was a little older than the average delivering mother (1.5 years older relative to 2014, Statistics Denmark – data not shown), had longer education and were more often predisposed to atopy. Thus, there is a risk of sample selection bias and the study results may not extend to the broader population.
We used BCG strain 1331 from the Danish State Serum Institute. Previous studies have shown that the immunogenicity of various BCG strains can differ substantially.[2] Therefore the present results may not extend to other strains of BCG.
Because it was only feasible to blind the investigators during data collection, the possibility of performance bias concerning parent reported outcomes with a subjective quality (Paper 1 and 3) cannot be ruled out, as the parents were well versed in the hypotheses for the Danish Calmette Study, and thus parents of vaccinated children may have had a different threshold for perceiving the child to be sick and for taking the child to the GP for example. This would tend to overestimate the effect of BCG.
Concerning parent reported infections, we only asked about a subset of common childhood infections and only recorded the number of episodes, not duration. Thus, we are not able to draw conclusions about other diseases or the length of each episode of illness. Also, parents in the control group possibly had less attachment to the data collection in the study and may thus underreport infections and GP visits whereby the effect of BCG would be diluted (Paper 1).
Concerning growth, body composition, and neurodevelopment, early life events that impact these may not manifest until decades later[46,73,74], something the short follow-up for this study cannot address (Paper 2 and 3). We did not have a reference for the correlation between skinfold thickness and body fat mass in our population, thus it is not known to what degree skinfolds are able to measure body fat mass in our population, but more sophisticated techniques yielding more easily interpretable results were not feasible in this setup (Paper 2).
Concerning child psychomotor development, we had a substantial rate of missing data, potentially biasing the effect of BCG towards the null. Recruiting premature children to the study proved a challenge and only approximately half of the intended number were enrolled, yielding a very low power in this subgroup. Also, due to a technical error we did not have ASQ at 6 months on the children born at GA < 33 weeks and 4 days (Paper 3).

6.3 Statistical considerations

Our study addresses a wide range of interrelated outcomes (Paper 1, 2, and 3 and Figure 2). This raises the issues of multiple testing and independence of null hypotheses. Multiple testing increases the risk of type 1 errors, which can be countered by adjusting the p-values for the number of tests performed.[75] The decision on whether or not to perform multiple testing adjustments to the p-values depend, among other things, on whether the trial is confirmatory and results are going to be used directly for decision making, or whether it has an exploratory character.[76] The Danish Calmette Study was the first of its kind with these outcomes in this particular setting, which differ substantially from previous BCG trials, and could thus be seen as exploratory in its nature, but on the other hand, the outcomes, subgroups, and statistical analyses[77] were chosen a priori, deliberately and, for the primary outcome of the Danish Calmette Study (all-cause hospitalizations), with the intent of being relevant to public health.(Stensballe et al. 2016a)
We used the Holland method of correction which keeps the familywise error rate (FWER) under control.[62] In order to perform adjustments for multiple testing, it is necessary to define the number of tests that belong to each 'experiment' in order to be able to control the FWER. In this thesis, from a statistical point of view, each paper has been considered a separate experiment, with subgroups as exploratory outcomes, thus keeping the FWER under control for the main and secondary outcomes of each paper.
When analyzing multiple interrelated outcomes from a single intervention, another approach to multiple testing is to keep the false discovery rate (FDR) under control.[78] The philosophy of this approach is that it keeps the risk of each rejected null hypothesis being true constant, and thus gains more power at the expense of an increased number of type 1 errors. The idea is that the goal is not to make sure that no type 1 errors are made in the individual analyses, but that the overall conclusion from evaluation of all the analyses has the least risk of leading to errors, either type 1 or type 2.[78] That is, if it is expected that a certain fraction of the null hypotheses are false, then one would be less concerned with a few type 1 errors if the goal is to draw overall conclusions regarding whether an intervention has an effect at all, and less concern is given to which of the interrelated outcomes the effect is on.[78,79]
In our case, another choice of method for multiple testing adjustment would not have changed the interpretation of the data in this thesis: The only p-value that was below 0.05 prior to adjustment (decreased incidence of visits to the GP from 0 to 3 months among BCG vaccinated children, unadjusted p-value = 0.02, Paper 1) would become statistically non-significant regardless of method (FWER[Holland]-adjusted p-value = 0.22, FDR[Simes]-adjusted p-value = 0.25, data not shown).
The analysis plan stated that the secondary outcomes of the papers in this thesis should be subject to multiple testing corrections. Since none of the statistical analyses in Paper 2 or 3 had a p-value below 0.05, multiplicity adjusted p-values were not reported in these papers.
If sub-group analyses are predefined and used for confirmation of previous hypotheses, the p-values only need to be multiplicity adjusted in order to be interpretable and keep within the FWER.[76] Since the sub-groups were pre-specified, they could be seen as confirmatory in which multiple testing adjustment of the p-values would be in place, but particularly the maternal BCG subgroup could arguably be seen as exploratory, since it was based on the observation that another vaccine (measles vaccine[61]) elicited greater non-specific protection against infections if given in the presence of maternal antibodies. Thus controlling for multiplicity could give inflated confidence[76] in the finding if it remained statistically significant, instead of just reporting the raw data and let the reader interpret the analysis as exploratory. In the papers in this thesis, subgroup analyses have not been adjusted for multiple testing and thus the reader should

keep the risk of type 1 error in mind when interpreting the results.

6.4 Ethical considerations

In order to conduct a trial where subjects are randomly assigned to a treatment according to present ethical standards, it is paramount that there is equipoise as to whether or not the treatment will be beneficial overall.[80,81] The case can be made that this applies to the Danish Calmette Study. Most of the previous evidence on BCGs non-specific prevention of infections comes from human studies and indicates a beneficial effect, but in the Danish Calmette Study it is applied in a new context, where the same effects may not be found. Conversely, the possible detrimental effects are derived mostly from theoretical considerations based primarily on animal studies (Figure 2). Thus it could not be predicted whether there would be any overall effects on the intervention group, and if there were, whether they would be beneficial or detrimental, and thus equipoise can be argued to have been present prior to study start.

7. CONCLUSION AND FUTURE PERSPECTIVES

The Danish Calmette Study is the first large, high-quality randomized trial that has been conducted in a high-income setting evaluating the non-specific effects of BCG. The papers in this thesis do not provide evidence of a public health benefit concerning prevention of infections in a high-income setting, in contrast to the current evidence in low-income settings. One possible explanation for this could be the lack of exposure to BCG or environmental mycobacteria among the mothers, as suggested by a subgroup analysis that indicated a protective effect against infections in children if the mother was BCG vaccinated. A subgroup analysis by Stensballe (Stensballe et al. 2016b) indicated a detrimental non-specific effect on infections among premature children, which was corroborated by a non-significant tendency in Paper 1. These findings should be explored further in other studies.
Reassuringly, BCG did not affect either growth or development of the overall population during the follow-up for our study. Extrapolation to the absence of later effects should be done with care, as other early life inflammatory events may affect health decades later.[74,82]
Drawing firm conclusions on the basis of a single trial in a specific setting is probably not justified, especially if the result is surprising given the prior evidence. When no effect was found, there is always the risk of accepting a false null hypothesis. Currently there is another BCG trial running in Australia, testing whether BCG can reduce the incidence of allergic illness and infections. The results of the Australian trial will hopefully be able to corroborate or contradict some of the unexpected findings from The Danish Calmette Study.
A BCG trial has also been conducted in Uganda.[83] Data collection has recently finished and results are expected during the fall of 2016. The outcomes of that trial include immune mechanisms and infectious illness within the first 6 to 10 weeks of life.

8. SUMMARY

The Bacillus Calmette-Guérin vaccine (BCG), which is used to protect against tuberculosis, has been associated with a variety of other effects since it was developed almost 100 years ago. Most notably, observational studies and randomized clinical trials from low-income countries indicate that it protects against unrelated infections, i.e. a so-called non-specific effect. The Danish Calmette Study was conducted to study these effects in a high-income population.
The immune response to BCG is not fully understood but involves a pro-inflammatory profiling of the immune system, also when exposed to unrelated pathogens. Immune changes have been implicated in changes in both child growth and child development and for that reason we also studied these outcomes.
We randomized 4262 children at birth to receive BCG vaccination at birth or to a no-intervention control group. We had prespecified subgroup analyses of child sex, prematurity, and maternal BCG vaccination. The statistical analysis plan was finalized prior to unblinding of the data.
Follow-up for the outcomes reported in this thesis consisted of telephone interviews and clinical examination at age 3 and 13 months, as well as online developmental questionnaires distributed to the parents at 12 months and additionally to the parents of premature children at age 6 and 22 months. The outcomes of this thesis were number of parent reported infections, child growth and body composition, and child psychomotor development.
Overall, there was no effect of BCG on either incidence of infections, growth, body composition or psychomotor development. A subgroup analysis of children of mothers who were BCG vaccinated showed a reduced incidence of infections from 0 to 3 months among BCG vaccinated children (incidence rate ratio = 0.62, CI: 0.39 to 0.98), but there was no effect from 3 to 13 months. Previous research has shown that maternal exposure to BCG or mycobacteria can alter the effect of BCG in the offspring, and thus the unexpected lack of effect on overall infections can possibly be explained by the lack of maternal exposure to BCG in our study, as only 17% of the mothers were BCG vaccinated. In the studies where non-specific protective effects of BCG have previously been found, most of the mothers were BCG vaccinated or exposed to mycobacteria.
Premature children had a non-significant increased risk of infection, which was corroborated by an analysis of hospitalizations for infections (not reported in this thesis). This was also unexpected as previous research indicated a more beneficial effect among low birth weight children. The study did not have power enough to exclude a negative effect of BCG on the development of premature children, and thus a cautious approach to vaccinating premature children may be prudent in a high-income setting.
We succeeded in recruiting the planned number of participants and had high follow-up rates for most outcomes. A limitation is that it was not feasible to blind the parents to the randomization group.
In conclusion, BCG did not have any public health benefit on incidence of infections and did not affect child growth or child development in the present study.

ABBREVIATIONS

Acronym	Definition
ASQ	Ages and Stages Questionnaire
BCG	Bacillus Calmette-Guérin
CI	95% Confidence interval
CNS	Central nervous system
FDR	False discovery rate
FWER	Familywise error rate
GA	Gestational age
GP	General practitioner
IL	Interleukin
IRR	Incidence rate ratio

Acronym	Definition
MUAC	Mid upper-arm circumference
NA	Not applicable
SD	Standard deviation
SSI	Statens Serum Institut
TNF	Tumor necrosis factor
WHO	World Health Organization

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