OPEN
Odense Patient data Explorative Network

Statistical Analysis Plan (SAP)

BCG-DENMARK-ELDERLY

Part A:

Using BCG vaccine to enhance non-specific protection of senior citizens during the COVID-19 pandemic. A randomized clinical trial (BCG-DENMARK-SENIOR).

Part B:

Using BCG vaccine to strengthen the immune system in the elderly and improve the response to influenza vaccine. A randomized clinical trial (BCG-DENMARK-INFLUENZA).

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1.0 ADMINISTRATIVE INFORMATION, Part A

Title	Using BCG vaccine to enhance non-specific protection of senior citizens during the COVID-19 pandemic. A randomized clinical trial (BCG-DENMARK-SENIOR).
Trial registration	EudraCT number 2020-003904-15
SAP version and date	1.0, 19-05-2023
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2.0 INTRODUCTION

Background

Worldwide, the population of persons > 60 years of age is growing because of advances in average life expectancy, especially in high income countries[1]. Due to age related decline in immune function, so called immunosenescence, the elderly are at higher risk of infectious diseases[2]. Severe infections are more common in the elderly, and they are more likely to be hospitalized due to complications related to infectious diseases. During the COVID-19 pandemic, it has become clear that elderly people are particularly susceptible to severe COVID-19. Strategies to strengthen senior citizens' immune system are urgently warranted.

The Bacille Calmette Guérin (BCG) vaccine was developed for tuberculosis prevention, but there is increasing evidence that it also has beneficial heterologous "non-specific" effects on the immune system[3–5]. Prior studies have shown that BCG vaccination strengthens the innate immune system resulting in increased activity against non-related infections[6–8]. BCG vaccination may strengthen the immune system of senior citizens and may (partially) protect against getting infected and/or experiencing severe morbidity due to infections in general and SARS-CoV-2 in particular[9,10].

We hypothesize that BCG vaccination can reduce the risk of COVID-19 and other infections among senior citizens during the COVID-19 pandemic[11]. A randomized controlled trial provides the highest validity for this research question.

Objectives and hypothesis

Primary objective: To reduce senior citizens' risk of acute infection during the COVID-19 pandemic.

Secondary objectives: To reduce senior citizens' risk of SARS-CoV-2 infection during the COVID-19 pandemic and to reduce self-reported respiratory illness in general during the COVID-19 pandemic.

The hypothesis is that BCG vaccination of seniors will reduce the risk of acute infection by 25% over a period of 12 months.

3.0 STUDY METHODS

Trial design

A single-blinded, placebo controlled randomized clinical trial. Participants are randomized 1:1 to BCG or placebo and will be followed for 12 months post-randomization with respect to illness, medical contacts, use of antibiotics, hospitalization, and death. The follow-up takes place both through self-reporting and through the Danish National Registers. The participants will receive an electronic questionnaire biweekly with questions about illness, medical contacts, other vaccinations, and side effects.

People ≥ 65 years of age are eligible for participation.

Randomization

Randomization takes place at baseline after informed consent is given. The randomization is done in varying blocks of 6-8, stratified by sex and age group (65-74; 75+) using REDCap electronic data capture tools hosted at the Region of Southern Denmark[12,13].

Sample size

With an expected incidence of "acute infection" of 20% we will be able to show a 25% reduction in the risk of acute infection in the intervention group compared to the placebo group by including a total of 1890 individuals: 945 individuals in each group. Since the primary outcome data to a large extent are obtained via national registers, we anticipate limited loss of power due to loss to follow-up. We therefore aimed to enroll 1900 participants.

Due to the pandemic and varying infectious disease precautions, recruitment took longer than anticipated, and we ended up recruiting a total of 1706 participants (90% of the anticipated sample size) between September 2020 and December 2021.

Timing of final analyses and outcome assessments

All outcomes are analyzed after end of trial.

4.0 STATISTICAL PRINCIPLES

Levels of confidence and p-values

A double-sided p-value of < 0.05 will be considered statistically significant. 95% confidence intervals will be provided.

Protocol violations

Protocol deviations and exclusions will be reported for each treatment arm.

5.0 STUDY POPULATION

Screening data

Around 35,000 elderly > 65 years live in Odense at the time of recruitment. The study is based on voluntary participation open to all eligible persons on their own initiative. Interested persons are screened for participation after they have received oral and written information and declared their continued interest for participation. We keep a list of persons screened for participation and reasons for not being included/being excluded.

Recruitment

Recruitment, enrolment, and follow-up with collection of biological material is conducted at activity houses for senior citizens run by the Municipality of Odense. The project is presented at special arrangements at these houses and advertised in local media and via posters in relevant public places.

Exclusion/loss to follow-up

Participants who are excluded after randomization or lost to follow up are not replaced. Participants are under follow-up for 12 months post-randomization or until they withdraw their consent, migrate to another country, or die.

Baseline characteristics, study population

Continuous baseline characteristics will be reported as mean with standard deviation or median with inter-quartile range, as appropriate. Categorical baseline characteristics will be reported as count and percentage. No statistical testing for baseline characteristics will be performed.

6.0 DESCRIPTION OF OUTCOMES AND VARIABLES

Primary outcome

The primary outcome is a composite outcome: acute infection identified either by a doctor, by antibiotics use, by hospitalization, or by death due to infection. All subcomponents will also be presented separately.

Information on hospitalizations, deaths, and antibiotics use is retrieved from national health registers: The Danish National Patient Register, The Central Person Register, and The National Prescription Register. An acute infection event identified by a doctor is defined as newly onset infectious illness that has prompted the participant to seek medical attention from general practitioner (GP) or on-call doctor and is based on self-reporting. Participants are asked in the questionnaires, if they have needed to see their GP or an on-call doctor due to newly onset illness during the past 14 days (yes/no). Thus, participants can only have one such event per survey period (14 days). For all outcomes, a honeymoon period of 14 days will be used to define new events.

Secondary outcomes

Secondary outcomes are verified SARS-CoV-2 infection (first event) and self-reported respiratory infection. Information on positive test results will be retrieved from the Danish Health Data Authority (Sundhedsdatastyrelsen) and information on self-reported respiratory infections will come from the follow-up questionnaires.
Verified SARS-CoV-2 infection is defined as having a positive SARS-CoV-2 PCR (Polymerase Chain Reaction), rapid antigen test, or converting from negative to positive antibody test (IgG positive) without having been vaccinated. As a supplementary analysis, we will explore the effect of BCG on severity of COVID-19. For this purpose, we divide verified SARS-CoV-2 infections into the following categories:

- Asymptomatic: Having a positive test result but no symptoms consistent with COVID-19 within the next 14 days.
- Mild: Having symptoms of COVID-19 (e.g., fever, cough, sore throat, malaise, headache, muscle pain, nausea, vomiting, diarrhea, loss of taste and smell) but not needing hospitalization.
- Severe: Lower respiratory infection (pneumonia), general deterioration, or respiratory failure requiring hospitalization.

Self-reported illness is defined as a respiratory infection if the participants report having had a respiratory infection such as common cold, influenza, pneumonia or similar term, or they report having had one or more of the following symptoms: cough, sore throat, runny nose/nasal congestion (common cold symptoms), loss of smell or taste sense, or dyspnea

with or without general symptoms such as fever, chills, muscle ache, headache, and fatigue (dyspnea only if in combination with fever).

Variables

Variable	Definition	Type	Scale
Primary outcome:			
Acute infection	Death, hospitalization, antibiotic treatment, or visit to GP due to newly onset infectious disease. Composite outcome.	Categorical, binary	0,1
Death due to infection	Death due to infectious disease.	Categorical, binary	0,1
Hospitalization for infection	Hospitalization due to infectious disease.	Categorical, binary	0,1
Antibiotic treatment	Antibiotic treatment during follow-up.	Categorical, binary	0,1
Doctor diagnosed infection	Infection identified by GP or on-call doctor.	Categorical, binary	0,1
Secondary outcomes:			
Self-reported respiratory infection	Reported respiratory symptoms or disease compatible with respiratory infection.	Categorical, binary	0,1
Verified CoV[1] infection (first event)	Positive SARS-CoV-2 test.	Categorical, binary	0,1
Asymptomatic COVID-19	Positive CoV test, no symptoms.	Categorical, binary	0,1
Mild COVID-19	Positive CoV test, mild symptoms, not hospitalized due to COVID-19.	Categorical, binary	0,1
Severe COVID-19	Positive CoV test, hospitalized due to COVID-19.	Categorical, binary	0,1

[1] SARS-CoV-2 infection.

7.0 ANALYSIS

Methods

All analyses will be performed from the intention-to-treat principle, for all participants studied together and stratified by sex and age group.

Several couples participated in this study. To consider shared vulnerability, we will cluster on couples (non-stratified analysis).

Primary outcome:

Acute infection will be analyzed as a recurrent time-to-event using an Andersen-Gill Cox proportional hazards regression model with time since inclusion as underlying time scale. The proportional hazards assumption will be tested using Schoenfeld residuals. The analysis will be done for the composite outcome and for all the subcomponents separately presenting hazard ratio with 95% confidence intervals for each. Absolute risk reductions will be calculated using Cox regression.

Secondary outcomes:

The secondary endpoint self-reported respiratory infection will be analyzed the same way as the primary endpoint. As participants are only able to report one infectious episode during a follow-up questionnaire, the event will be assigned to the first day within the questionnaire period, and the following 14 days of follow-up excluded for analyses as it represents risk free time.

The secondary outcomes verified SARS-CoV-2 infection and severity of COVID-19 will be analyzed in a standard Cox proportional hazards model, but otherwise as described above.

Supplementary analyses

We will conduct preplanned supplementary analyses to assess possible effect modification by other vaccines given during follow-up, previous BCG vaccination, and COVID-19 vaccination before inclusion. These will be done using the same methods as described above for the primary (composite) outcome and for both secondary outcomes.

1. Before and after receipt of subsequent vaccines, and adjusted by type of subsequent vaccine, to assess possible interaction between BCG and other vaccines. Vaccination status will be categorized as 1) No other vaccines, 2) COVID 19 vaccine, 3) Influenza vaccine, or 4) COVID 19 and influenza vaccine.

Vaccination status will be included as a time varying covariate changing on the date of vaccination with subsequent vaccines received during follow-up. Some participants received a pneumococcal vaccine (23-valent polysaccharide vaccine) together with their influenza

vaccine as was recommended by the Danish Board of Health in 2020. We will therefore include influenza vaccines with or without concurrent pneumococcal vaccination (given on the same day). Participants will be censored upon receipt of any other vaccines than the above mentioned.

2. By previous BCG vaccination (Yes/no, self-reported and assessed by BCG scar) to assess the effect of revaccination vs. first vaccination with BCG.

3. By COVID-19 vaccination status at inclusion (Received COVID 19 vaccines yes/no).

Exploratory analysis

Participants were enrolled during different stages of the COVID-19 pandemic. We will therefore conduct a subgroup analysis of all outcomes comparing participants included in the same phase of the pandemic. We will compare outcomes for participants included in the following periods:

1. September 2020 to January 2021 (N=1239); before the COVID-19 vaccines were widely distributed to the target population. A period characterized by increasing infection control measurements and concluding in lockdown.
2. April to December 2021 (N=438); Participants are now being vaccinated with the new coronavirus vaccines; most have had the two or three doses before enrollment. A period characterized by varying COVID-19 activity; a minimum of restrictions on society despite periodically high transmission rates (Omicron variant in the fall).

Missing data

Participants are under follow-up for the primary outcome irrespective of missing questionnaires as this is mostly based on register data.

For the outcomes solely based on self-reported information (self-reported respiratory infections and severity of COVID-19) participants are only under follow-up during the periods where they have completed follow-up questionnaires.

Adverse events

Adverse events will be described in terms of incidence in the groups and more thoroughly presented in a table as part of supplementary material (Table 4). Serious adverse events

(SAE) will be classified as related to study drugs (possible/probable/definite) or unrelated to study drugs (unlikely/doubtful). If related, they are classified as expected/unexpected (SAR/SUSAR).

8.0 SUPPLEMENTARY INFORMATION

Figures and tables

Figure 1: Flow chart, inclusion.

Table 1: Baseline characteristics.

Table 2: Results primary and secondary outcomes.

Table 3: Supplementary analyses (sex, age group, other vaccines, previous BCG, COVID-19 vaccination status at inclusion).

Table 4: Adverse events and serious adverse events.

Table 5: Short description of serious adverse events.

Figure 1: Flow chart, inclusion

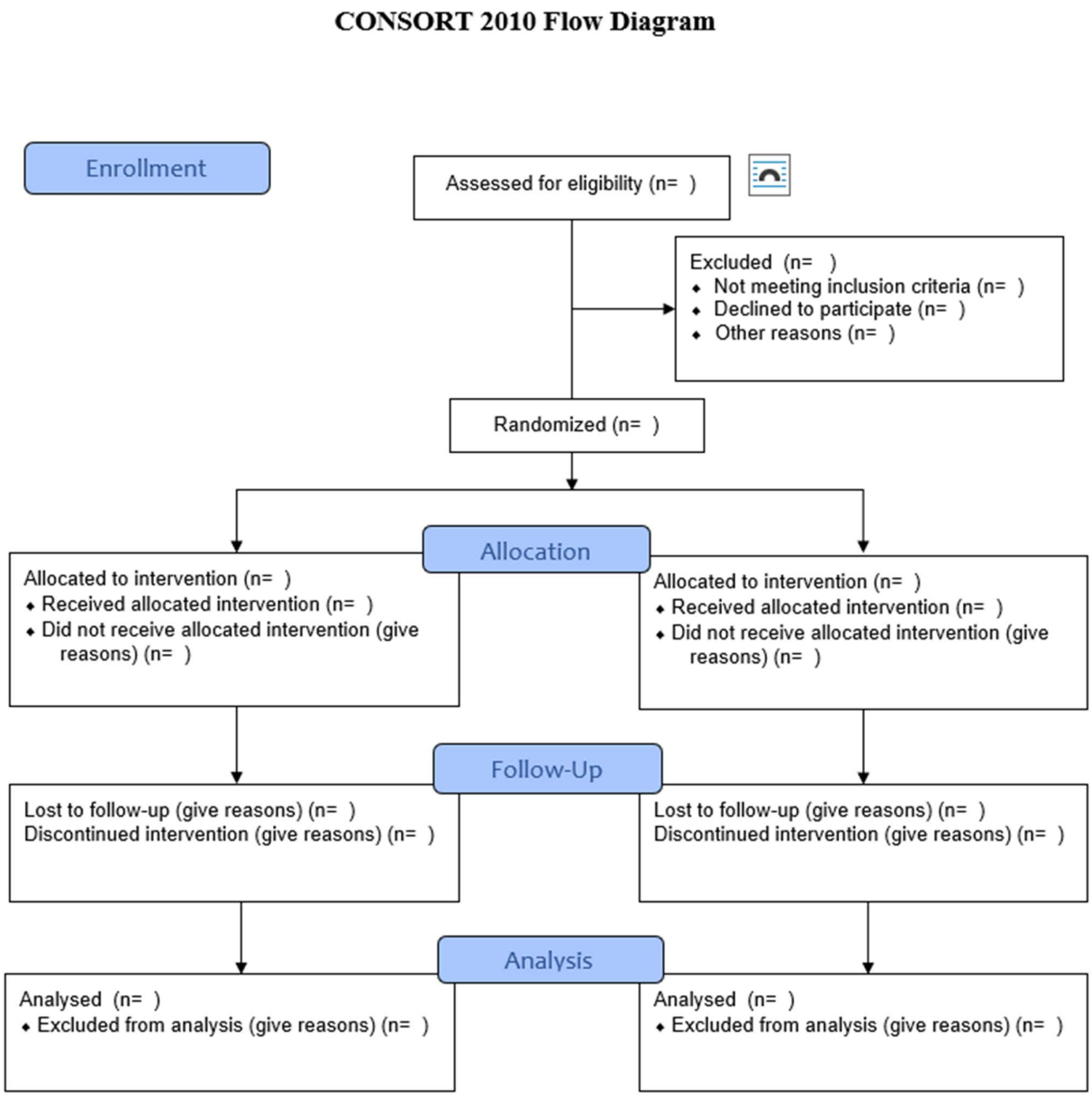


Table 1 Baseline characteristics

	BCG	Placebo
Age, median (IQR)		
Sex female (%)		
Living alone (%)		
Spouse included in study (%)		
Active smoker (%)		
Previous smoker (%)		
Alcohol consumption, units per week, mean (SD)		
BMI, median (IQR)		
Chronic diseases		
Cardiovascular (%)		
Diabetes (%)		
Lung (%)		
Other (%)		
Level of education		
Low (%)		
Medium (%)		
High (%)		
Previously BCG vaccinated (%)		
Scar from BCG vaccination observed (%)		
COVID-19 vaccinated, yes (%)[1]		
One dose		
Two doses		
Three doses		

[1] COVID-19 vaccinated before enrollment.

Table 2 Results primary and secondary outcomes

Outcome	Number (%)		Hazard ratio (95% CI)	Absolute risk reduction (95% CI)
	BCG *N=xxx*	Placebo *N=xxx*		
Primary outcome				
Acute infection	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Death	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Hospitalization	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Antibiotics	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Doctor	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Secondary outcomes				
Self-reported respiratory infection	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Verified COVID-19	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
COVID-19 severity				
Asymptomatic	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Mild	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Severe	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)

Table 3 Supplementary analyses to explore possible effect modification by sex, age group, other vaccines, previous BCG, and COVID-19 vaccination status at inclusion.

	Number (%)		Hazard ratio (95% CI)	Absolute risk reduction (95% CI)
	BCG *N=xxx*	Placebo *N=xxx*		
Acute infection – also where numbers permit subdivided on each of the outcomes included in the composite outcome.				
Females	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Males	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age < 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age ≥ 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Other vaccines received after randomization:				
Effect of intervention before other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect of intervention after other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect after other vaccines, by type of vaccine received:				
COVID vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Influenza vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Both vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Has BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
CoV vaccinated before enrollment:				
Yes	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 1[2]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 2[3]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)

Self-reported respiratory infection				
Females	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Males	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age < 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age ≥ 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Other vaccines received after randomization:				
Effect of intervention before other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect of intervention after other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect after other vaccines, by type of vaccine received:				
COVID vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Influenza vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Both vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Has BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
CoV vaccinated before enrollment:				
Yes	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 1[2]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 2[3]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Verified COVID-19				
Females	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Males	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age < 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Age ≥ 75	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)

Other vaccines received after randomization:				
Effect of intervention before other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect of intervention after other vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Effect after other vaccines, by type of vaccine received:				
COVID vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Influenza vaccine	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Both vaccines	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Has BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No BCG scar[1]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
CoV vaccinated before enrollment:				
Yes	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
No	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 1[2]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Included period 2[3]	xx (x.x)	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)

[1] Scar from previous BCG vaccination at enrollment.

[2] Period 1: September 2020 to January 2021 (N=1239); before the COVID-19 vaccines were widely distributed to the target population. A period characterized by increasing infection control measurements and concluding in lockdown.

[3] Period 2: April to December 2021 (N=438); Participants are now being vaccinated with the new coronavirus vaccines; most have had the two or three doses before enrollment. A period characterized by varying COVID-19 activity; a minimum of restrictions on society despite periodically high transmission rates (Omicron variant in the fall).

Table 4 Adverse Events (AE) and Serious Adverse Events (SAE)

Endpoint	**BCG**	**Placebo**
	Number (%) [M/F]	*Number (%) [M/F]*
Any SAE		
Hospitalization for any reason		
Death due to any reason		
Adverse events (AE):		
Injection site reaction		
Minor abscess/wound		
Severe local reaction (abscess)		
Fever		
Fatigue		
Headache		
Lymphadenitis		
Serious Adverse Events (SAE):		
Blood or lymphatic disorder		
Cardiac disorders		
Endocrine disorders		
Gastrointestinal disorders		
Infection		
Musculoskeletal and connective tissue disorders		
Nervous system disorders		
Respiratory disorders		

Urological and gynecological disorders		
Unknown/other		
Outcome SAE:		
Life threatening		
Required/prolonged hospitalization		
Resulted in death		

Table 5 Short description of Serious Adverse Events (SAE)

No	Category	Outcome	Description	Group[1]	Sex	Week[2]	Before/after[3]
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

[1]Randomisation group.

[2]Weeks after inclusion.

[3]Occurrence before or after other vaccines were administered to the participant.

Statistical Analysis Plan (SAP)

1.0 ADMINISTRATIVE INFORMATION, Part B	
Title	Using BCG vaccine to strengthen the immune system in the elderly and improve the response to influenza vaccine. A randomized clinical trial (BCG-DENMARK-INFLUENZA).
Trial registration	EudraCT number 2019-002781-12
SAP version and date	1.0, 19-05-2023
Protocol version and date	5.6, 07-10-2021
SAP contributors	Anne Marie Rosendahl Madsen (PI) Lise Gehrt (Data analyst) Ola Løvsletten (Statistician) Christine Stabell Benn (Sponsor)
Sponsor	Christine Stabell Benn University of Southern Denmark, Department of Clinical Research and Bandim Health Project
Primary investigator	Anne Marie Rosendahl Madsen University of Southern Denmark, Department of Clinical Research and Bandim Health Project
Trial statistician	Ola Løvsletten The Arctic University of Norway, Tromsø Department of Community medicine
SAP revision history	NA
Date of approval	19-05-2023 (all authors).

2.0 INTRODUCTION

Background

Severe influenza virus infections are more common in the elderly and seasonal vaccination with inactivated influenza vaccine (IIV) is recommended to persons above 65 years of age[14]. However, due to immunosenescence, the efficacy of vaccines decrease with increasing age[15,16].

The Bacille Calmette Guérin vaccine (BCG) was developed for tuberculosis prevention, but there is increasing evidence that it also has beneficial heterologous "non-specific" effects on the immune system[3,5,17]. Prior studies have shown that BCG vaccine strengthens the innate immune system resulting in increased activity against non-related infections[7,9,18]. A potentiating effect of BCG vaccine on the response to other vaccines has also been observed in previous studies[19–21].

In the present project, we will explore BCG as a low-cost, low-risk intervention to increase the immune response to influenza vaccination and improve the general resistance towards infections in senior citizens.

Objectives and hypothesis

We will test the effects of BCG vaccination on the specific immune response to IIV and on the immune system and health in general in people ≥ 65 years, with the aim to:

- Improve their specific antibody response to influenza vaccination.
- Improve their general resistance towards infections.
- Study the effect of BCG on classical lymphocyte-dependent responses, and the induction of innate immune memory.

We will test the following hypotheses:

a) BCG vaccine given two weeks prior to, together with, or two weeks after IIV is associated with a 30% increase in seroconversion to IIV and/or a significant increase in influenza antibody titers.

b) The hypothesized potentiating effects of BCG is due to a combination of classical lymphocyte-dependent responses, and the induction of innate immune memory (trained

immunity) in myeloid cells.

10.0 STUDY METHODS

Trial design

A single-blinded, placebo controlled, randomized clinical trial, with nested immunological studies. Participants are randomized 1:1:1:1 to four groups: BCG 14 days before, together with, or 14 days after IIV compared to placebo.
People ≥ 65 years of age who plan to accept the offer of seasonal influenza vaccination are eligible for participation.

Participants are vaccinated once at day 0 and twice at day 14 (one shot in each arm). All participants receive the standard seasonal IIV recommended by the Danish Board of Health. The participants will come back for a follow-up interview at day 42 and all are followed for six months post-randomization with electronic questionnaires about their health. These include information on symptoms and disease, potential side effects, other vaccinations, visits to general practitioner or on call doctor, and hospitalizations. Every survey period is 14 days, starting from day 0 (inclusion).
Blood samples for influenza antibody testing are collected from all participants at day 14 and 42. Samples for further immunological analyses are collected from subgroups at day 0, day 14, day 21, and day 42.
Vaccination schedule:

Group	Day 0 (one shot)	Day 14 (two shots)	Day 42
1	BCG	IIV + Placebo	Follow up visit
2 (Control)	Placebo	IIV + Placebo	Follow up visit
3	Placebo	IIV + BCG	Follow up visit
4	IIV	Placebo + BCG	Follow up visit

Randomization

Randomization takes place at baseline after informed consent is given. The randomization is done in varying blocks of 6-8, stratified by sex and age group (65-74; 75+) using REDCap

electronic data capture tools hosted at the Region of Southern Denmark[12,13]. As described above, participants are randomized to one of the four groups and given the allocated treatment.

Sample size

We aim to include 75 participants in each group, 300 in total. With an anticipated seroconversion rate of 50% in the control group, we should be able to show a 30% increase in seroconversion rate associated with BCG vaccine with 80% power and an alpha of 0.05, anticipating 10% loss to follow-up.
All participants are followed for clinical symptoms for six months. With this sample size, we will only be able to show differences in clinical symptoms if such differences are pronounced.

Timing of final analyses and outcome assessments

All outcomes are analyzed after end of trial.

11.0 STATISTICAL PRINCIPLES

Levels of confidence and p-values

A double-sided p-value of < 0.05 will be considered statistically significant. 95% confidence intervals will be provided.

Protocol violations

Protocol deviations and exclusions will be reported for each treatment arm.

12.0 STUDY POPULATION

Screening data

As described for part A.

Recruitment

As described for part A.

Exclusion/lost to follow-up

Participants who are excluded after randomization or lost to follow up are not replaced. Participants are under follow-up for six months post-randomization or until they withdraw their consent, migrate to another country, or die.

Baseline characteristics, study population

Continuous baseline characteristics will be reported as mean and standard deviation or median and inter-quartile range, as appropriate. Categorical baseline characteristics will be reported as count and percentage. No statistical testing for baseline characteristics will be performed.

13.0 DESCRIPTION OF OUTCOMES AND VARIABLES

Primary outcome

Change in antibody levels to influenza virus strains included in the IIV, comparing levels at day 14 and day 42; just before and 4 weeks after influenza vaccination (for group 4, it will be 2 and 6 weeks after influenza vaccination).

We will use *Influvactetra* (Mylan ApS, Copenhagen), a quadrivalent inactivated influenza vaccine containing the following strains:

1. A/Victoria/2570/2019, IVR-215 (H1N1, pdm09-like strain)
2. A/Cambodia/e0826360/2020, IVR-224 (H3N2-strain)
3. B/Washington/02/2019, wild type
4. B/Phuket/3073/2013, wild type

Influenza antibodies are measured in hemagglutination-inhibition assay (HAI)[22] yielding dilution titers with an expected range of <20 to 320 for this population. Geometric mean titers (GMT) will be determined by calculating the mean of the log-transformed duplicate titers followed by back transformation (calculated as 10x , where x is defined as the mean log-transformed titer).

Seroconversion is defined as a pre-vaccination HAI titer <10 and a post-vaccination titer ≥ 40, or a pre-vaccination titer ≥ 10 and a minimum four-fold rise in post-vaccination titer[22].

Secondary outcomes

Self-reported infections:

Infections are identified through information given by the participants in the electronic follow-up questionnaires, where they are asked to describe disease episodes every 14 days. They are asked if they have been ill, and then asked to cross of their symptoms on a list. Furthermore, we ask them to provide the most likely cause for their illness.
Disease episodes are characterized as infections if participants report symptoms compatible with an infectious disease or they report an infectious disease diagnose as the cause of their illness. Criteria for self-reported infection:

- Having one or more of the following symptoms: fever, cough, sore throat, runny nose/nasal congestion (common cold symptoms), loss of smell or taste sense, vomiting, or diarrhea with or without general symptoms such as headache, myalgia, dysarthria, or fatigue.
- Having dyspnea AND fever with or without general symptoms such as headache, myalgia, dysarthria, or fatigue.
- Having described an infectious disease as the self-estimated cause of illness. This is a free-text field and participants answers will be classified as infectious disease or not by medically trained study personnel.

Baseline for analysis is set at day 14 reflecting completion of the allocated vaccinations. We will however also conduct the analysis from day 0-14. Participants can register a maximum of one infectious disease event per survey period (14 days).

A secondary analysis of association between influenza antibody level and subsequent risk of infection will be performed to test if high antibody titers correlate with risk of infection.

Immunological outcomes (subgroup analyses):

A random subset of participants (the first to accept) have extra blood samples taken for further immunological analyses.

1. Lymphocyte-dependent responses 7 days after influenza vaccination
2. The induction of innate immune memory 14- and 42-days post-randomization

For the lymphocyte analyses, 10 participants from groups 1-3 will have blood drawn on day 21, exactly 7 days after influenza vaccination. We will assess the effect of the different treatment combinations on the response to the influenza vaccine, examining activated

influenza antibody-secreting B-lymphocytes. Due to the different timing of the influenza vaccination, participants from group 4 are not part of this analysis.

For the trained immunity analyses, 15 participants from each group will have extra blood samples taken on day 0, 14, and 42. Peripheral blood mononuclear cells (PBMCs) are isolated from the samples and subsequently stimulated in vitro with various antigens. The immune function will be assessed by cytokine production capacity measured by Luminex technology after stimulation. The outcome measure for this analysis is concentration of different types of cytokines produced following stimulations. We will compare fold change in cytokine production between the groups at the various timepoints with and without adjustment for responses in control medium. This will allow us to determine whether BCG in the different combinations with influenza vaccine induces trained immunity in this population.

List of variables:

Variable name	Definition	Type	Scale
Primary outcome: Change in antibody levels to influenza virus strains included in the IIV, comparing levels at day 14 and day 42.			
ab_diff_strain	Influenza antibody titer (GMT) day 42 minus day 14. For each strain.	Continuous	<20 to 320
seroconv	Seroconverted after influenza vaccination (4-fold increase in Ab titer). For each strain.	Binary	0: no 1: yes
Secondary outcome, self-reported infections:			
infection	Infection or symptoms consistent with infection (0 or 1 every survey period).	Binary	0: no 1: yes
Secondary exposure:			
ab_day42_cat	Influenza ab titer day 42 (GMT). Secondary exposure.	Categorical	1. quartile 2. quartile 3. quartile 4. quartile
Secondary outcome, immunological:			
b_lymph	Number of activated B-cells. For all relevant subtypes.	Continuous	0.0-0.3 x 10^9/L

cytokine_stim_day	Cytokine concentration (TNFα, IL-1β, IFN-γ) after stimulation with: Poly(I:C), Pam3CSK4, LPS, and influenza vaccine at the various timepoints (day 0, 14, and 42).	Continuous	0-1500 pg/mL
diff_cytokine_stim_day	Fold change in cytokine concentration from baseline value (day 0) to day 14 and 42 after stimulation with various stimuli. Compared between the randomization groups. Using randomization group as exposure (categorical).	Continuous	0-10

14.0 ANALYSIS

Methods

All analyses will be performed from the intention-to-treat principle, for all participants studied together and stratified by sex and age group.

Primary outcome:

Mean change in GMT in samples from day 14 and 42 will be compared between the control group and the three treatment groups in linear regression analyses with adjustment for baseline level of GMT, age group, and sex. In case residuals are not normally distributed, the analysis will be performed on log-10 transformed change in GMT instead. The proportion of participants who seroconvert will be compared in logistic regression analysis with adjustment for age group and sex.

Secondary outcomes:

Self-reported infections:

The hazard ratio of infections will be estimated using an Andersen-Gill Cox proportional hazards regression model with infections included as recurrent events and time since inclusion as underlying time scale. The proportional hazards assumption will be tested using Schoenfeld residuals. As participants are only able to report one infectious episode during a follow-up questionnaire, the event will be assigned to the first day within the questionnaire period, and the following 14 days of follow-up excluded for analyses as it represents risk free time.

Association between influenza antibody titer level at day 42 and rate of infections will also be explored in Andersen-Gill Cox proportional hazards regression model, using antibody titer at day 42 as exposure and self-reported infections after day 42 as outcome. Antibody titers will be categorized into quartiles or similar relevant categories. The analysis will be adjusted for sex and age group.

Absolute risk reduction will be calculated using Cox regression.

Immunological outcomes (subgroup analyses):
Mean change in number of activated B-cells, concentration of cytokines, as well as fold change in cytokine concentration after stimulation from day 0 to day 14 and from day 14 to day 42 will be compared between the control group and the three treatment groups in linear regression analyses. With and without adjustment for responses in the control condition.

Missing data

Participants will be included in the analysis of the primary outcome irrespective of missing questionnaires as this is based on laboratory outcomes only. Participants with missing or inadequate blood samples on one of the sample days will be excluded from the antibody analysis.

For the secondary outcome (infection) which is based on self-reported information, participants are only under follow-up during the periods where they have completed follow-up questionnaires.

Adverse events

Adverse events will be described in terms of incidence in the randomization groups and more thoroughly presented in a table as part of supplementary material (Table 4). Serious adverse events (SAE) will be classified as related to study drugs (possible/probable/definite) or unrelated to study drugs (unlikely/doubtful). If related, they are classified as expected/unexpected (SAR/SUSAR).

15.0 SUPPLEMENTARY INFORMATION

Figures and tables

Figure 1: Flow chart, inclusion.

Figure 1: Flow chart, inclusion.

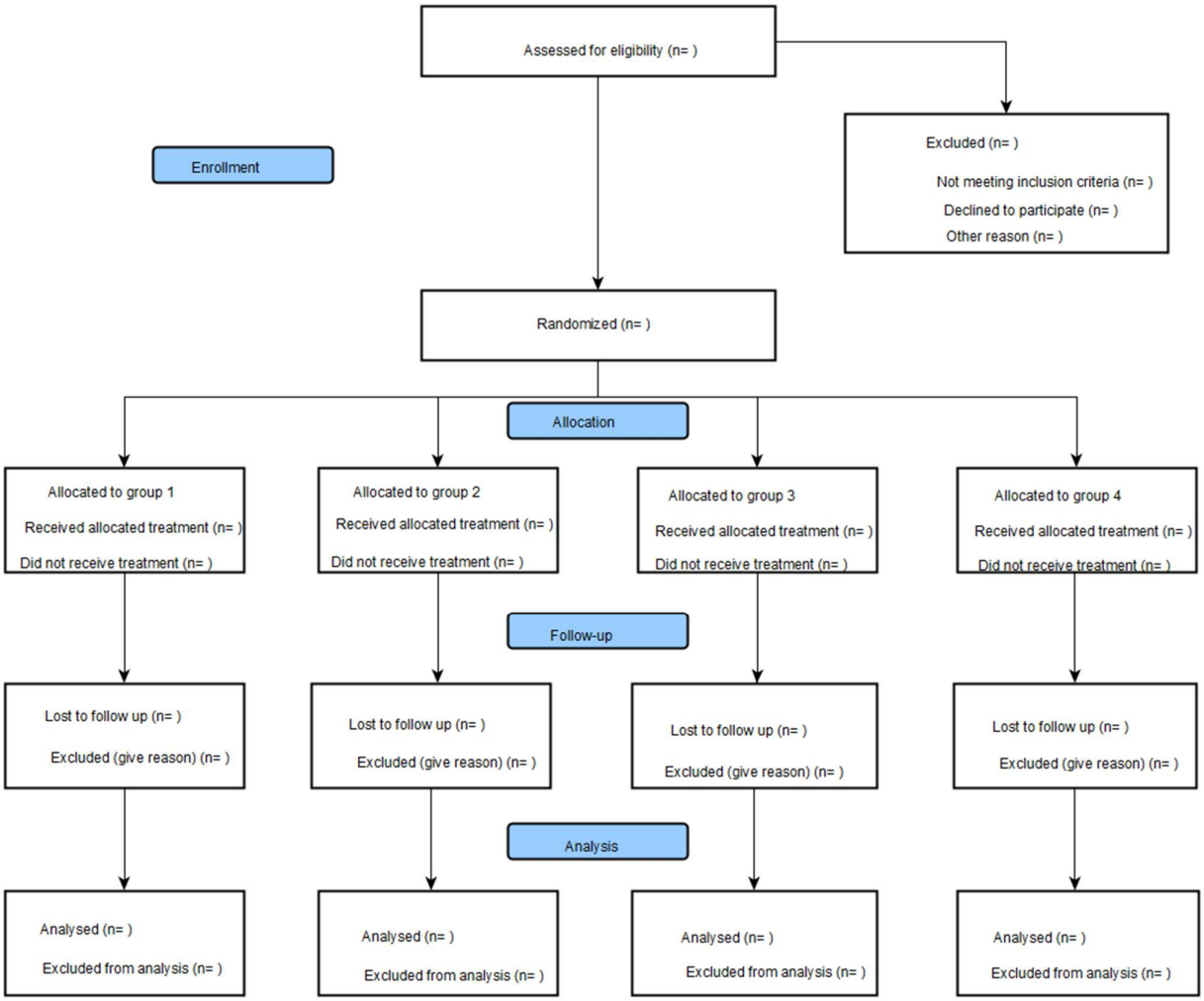


Figure 2: Study design.

A flow chart describing enrollment, follow-up visits, vaccinations, collection of blood samples, and surveys with a timeline.

Table 1: Baseline characteristics.

	Group 1	Group 2	Group 3	Group 4
Age, median (IQR)				
Sex female (%)				
Living alone (%)				
Spouse included in study (%)				
Active smoker (%)				
Previous smoker (%)				
Alcohol consumption, units per week, mean (SD)				
BMI, median (IQR)				
Chronic diseases				
Cardiovascular (%)				
Diabetes (%)				
Lung (%)				
Other (%)				
Level of education				
Low (%)				
Medium (%)				
High (%)				
Previously BCG vaccinated (%)				
Scar from BCG vaccination observed (%)				
COVID-19 vaccinated, yes (%)[1]				
One dose				
Two doses				
Three doses				

[1] COVID-19 vaccinated before enrollment.

Table 2: Results primary outcome.

Change in antibody levels to influenza virus strains included in the IIV, comparing levels at day 14 and day 42.

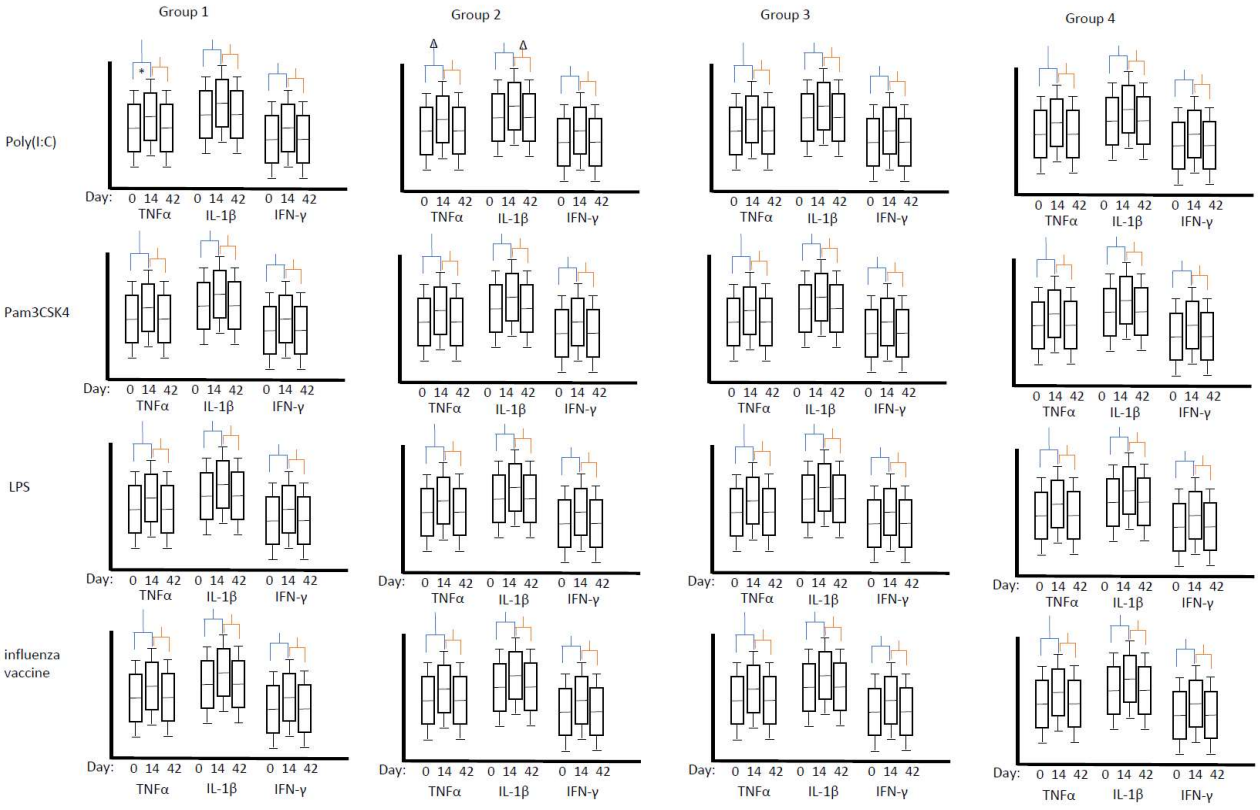
Influenza strain	Change in antibody titer from day 14 to day 42, mean (sd) β (95% CI)			
	Group 1	**Group 2 (Control)**	**Group 3**	**Group 4**
A/Victoria	xx (xx-xx) xx (xx-xx)	xx (xx) (ref.)	xx (xx) xx (xx-xx)	xx (xx) xx (xx-xx)
A/Cambodia	xx (xx) xx (xx-xx)	xx (xx) (ref.)	xx (xx) xx (xx-xx)	xx (xx) xx (xx-xx)
B/Washington	xx (xx) xx (xx-xx)	xx (xx) (ref.)	xx (xx) xx (xx-xx)	xx (xx) xx (xx-xx)
B/Phuket	xx (xx) xx (xx-xx)	xx (xx) (ref.)	xx (xx) xx (xx-xx)	xx (xx) xx (xx-xx)
	Seroconversion N (%) OR (95% CI)			
A/Victoria	xx (%) x.xx(x.xx–x.xx)	xx (%) (ref.)	xx (%) x.xx (x.xx–x.xx)	xx (%) x.xx (x.xx–x.xx)
A/Cambodia	xx (%) x.xx(x.xx–x.xx)	xx (%) (ref.)	xx (%) x.xx (x.xx–x.xx)	xx (%) x.xx (x.xx–x.xx)
B/Washington	xx (%) x.xx(x.xx–x.xx)	xx (%) (ref.)	xx (%) x.xx (x.xx–x.xx)	xx (%) x.xx (x.xx–x.xx)
B/Phuket	xx (%) x.xx(x.xx–x.xx)	xx (%) (ref.)	xx (%) x.xx (x.xx–x.xx)	xx (%) x.xx (x.xx–x.xx)

Table 3: Results infectious disease rate and association with influenza antibody level.

Treatment group	Number (%)	Hazard ratio (95% CI)	Absolute risk reduction (95% CI)
Self-reported infection six months post-randomization			
Group 1	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Group 2	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Group 3	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Group 4	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
Association between influenza antibody level at day 42 and infection rate during follow-up after day 42			
Antibody level by quartile	Number (%)	Hazard ratio (95% CI)	Absolute risk reduction (95% CI)
1 quartile	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
2 quartile	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
3 quartile	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)
4 quartile	xx (x.x)	x.xx (x.xx–x.xx)	x.x% (x.x%–x.x%)

Figure 3: Results trained immunity.

Boxplots showing fold increase in cytokine production after stimulation with bacterial antigens and influenza vaccine at day 0, day 14, and day 42 for each treatment group.



* Denotes within-group statistically significant change in cytokine levels between day 0 and 14 or between day 14 and 42
Δ Denotes between group statistically significant different change in cytokine levels between day 0 and 14 or between day 14 and 42

Figure 4: Results lymphocyte response.

Graphs showing numbers and or percentage of activated B-lymphocytes at day 21 per treatment group.

Table 4: Adverse events (AE) and Serious Adverse Events (SAE).

Endpoint	**Treatment group**			
	1	**2**	**3**	**4**
	Number (%) [M/F]			
Any SAE				
Hospitalization for any reason				
Death due to any reason				
Injection site reaction				
Minor abscess/wound				
Severe local reaction (abscess)				
Fever				
Fatigue				
Headache				
Lymphadenitis				
Blood or lymphatic disorder				
Cardiac disorders				
Endocrine disorders				
Gastrointestinal disorders				
Infection				
Musculoskeletal and connective tissue disorders				
Nervous system disorders				
Respiratory disorders				
Urological and gynecological disorders				

Unknown/other				
Life threatening				
Required/prolonged hospitalization				
Resulted in death				

Table 5 Short description of Serious Adverse Events (SAE)

No	Category	Outcome	Description	Group[1]	Sex	Week[2]	Before/after[3]
1							
2							
3							
4							
5							
6							
7							
8							
9							
10							

[1]Randomisation group.
[2]Weeks after inclusion.
[3]Occurrence before or after other vaccines were administered to the participant.

16.0 REFERENCES

1. United Nations, Department of Economic and Social Affairs, Population, Division . World Population Ageing 2015. 2015;(ST/ESA/SERA/390).
2. Crooke SN, Ovsyannikova IG, Poland GA, Kennedy RB. Immunosenescence and human vaccine immune responses. Immun Ageing 2019;16(1).
3. Higgins JPT, Soares-Weiser K, López-López JA, Kakourou A, Chaplin K, Christensen H, et al. Association of BCG, DTP, and measles containing vaccines with childhood mortality: Systematic review. BMJ 2016;355.
4. Kristensen I, Aaby P, Jensen H, Fine P. Routine vaccinations and child survival: follow up study in Guinea-Bissau, West Africa Commentary: an unexpected finding that needs confirmation or rejection. BMJ 2000;321(7274):1435–1435.
5. Benn CS, Fisker AB, Rieckmann A, Sørup S, Aaby P. Vaccinology: time to change the paradigm? Lancet Infect Dis 2020;20(10):e274–83.
6. Netea MG, Quintin J, Van Der Meer JWM. Trained immunity: A memory for innate host defense. Cell Host Microbe2011;9(5):355–61.
7. Netea MG, Joosten LAB, Latz E, Mills KHG, Natoli G, Stunnenberg HG, et al. Trained immunity: A program of innate immune memory in health and disease. Science (80-.).2016;352(6284):427.
8. [Kleinnijenhuis J, Quintin J, Preijers F, Joosten LAB, Ifrim DC, Saeed S, et al. Bacille Calmette-Guérin induces NOD2-dependent nonspecific protection from reinfection via epigenetic reprogramming of monocytes. Proc Natl Acad Sci U S A 2012;109(43):17537–42.
9. Giamarellos-Bourboulis EJ, Tsilika M, Moorlag S, Antonakos N, Kotsaki A, Domínguez-Andrés J, et al. Activate: Randomized Clinical Trial of BCG Vaccination against Infection in the Elderly. Cell 2020;183(2):315-323.e9.
10. Tsilika M, Taks E, Dolianitis K, Kotsaki A, Leventogiannis K, Damoulari C, et al. ACTIVATE-2: A Double-Blind Randomized Trial of BCG Vaccination Against COVID-19 in Individuals at Risk. Front Immunol 2022;13:873067.
11. O'Neill LAJ, Netea MG. BCG-induced trained immunity: can it offer protection against COVID-19? Nat Rev Immunol 2020;20(6):335–7.

12. Harris PA, Taylor R, Minor BL, Elliott V, Fernandez M, O'Neal L, et al. The REDCap consortium: Building an international community of software platform partners. J. Biomed. Inform.2019;95.
13. Harris PA, Taylor R, Thielke R, Payne J, Gonzalez N, Conde JG. Research electronic data capture (REDCap)-A metadata-driven methodology and workflow process for providing translational research informatics support. J Biomed Inform 2009;42(2):377–81.
14. Dorrington MG, Bowdish DME. Immunosenescence and novel vaccination strategies for the elderly. Front. Immunol.2013;4(JUN).
15. Goodwin K, Viboud C, Simonsen L. Antibody response to influenza vaccination in the elderly: A quantitative review. Vaccine 2006;24(8):1159–69.
16. Chen WH, Kozlovsky BF, Effros RB, Grubeck-Loebenstein B, Edelman R, Sztein MB. Vaccination in the elderly: an immunological perspective. Trends Immunol.2009;30(7):351–9.
17. Biering-Sørensen S, Aaby P, Lund N, Monteiro I, Jensen KJ, Eriksen HB, et al. Early BCG-Denmark and Neonatal Mortality among Infants Weighing <2500 g: A Randomized Controlled Trial. Clin Infect Dis 2017;65(7):1183–90.
18. Cirovic B, de Bree LCJ, Groh L, Blok BA, Chan J, van der Velden WJFM, et al. BCG Vaccination in Humans Elicits Trained Immunity via the Hematopoietic Progenitor Compartment. Cell Host Microbe 2020;
19. Libraty DH, Zhang L, Woda M, Acosta LP, Obcena A, Brion JD, et al. Neonatal BCG vaccination is associated with enhanced T-helper 1 immune responses to heterologous infant vaccines. Trials Vaccinol 2014;3(1):1–5.
20. Ritz N, Mui M, Balloch A, Curtis N. Non-specific effect of Bacille Calmette-Guérin vaccine on the immune response to routine immunisations. Vaccine 2013;31(30):3098–103.
21. Leentjens J, Kox M, Stokman R, Gerretsen J, Diavatopoulos DA, Van Crevel R, et al. BCG vaccination enhances the immunogenicity of subsequent influenza vaccination in healthy volunteers: A randomized, placebo-controlled pilot study. J Infect Dis 2015;212(12):1930–8.
22. Katz JM, Hancock K, Xu X. Serologic assays for influenza surveillance, diagnosis and vaccine evaluation. Expert Rev. Anti. Infect. Ther.2011;9(6):669–83.