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Age-dependent effect of BCG vaccination on the kinetics of biological aging

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Abstract

BCG vaccination has been reported to enhance immune responsiveness and attenuate systemic inflammation - processes closely linked to immune aging. Here, we extend the evaluation of BCG vaccination effects on biological aging by examining its effects on epigenetic age scores. We profiled genome-wide DNA methylation in two BCG-vaccinated cohorts: 284 young adults from the 300BCG study with samples collected at baseline, two weeks, and three months post-vaccination, and 117 older adults (> 65 years) from the BCG-DENMARK-SENIOR study with samples obtained at baseline and one year after vaccination. BCG vaccination exerted complex, age- and sex-dependent effects on epigenetic aging. In young individuals, particularly men, BCG vaccination significantly reduced epigenetic age acceleration, which was accompanied by a parallel decrease in an inflammatory age score. In contrast, in older adults, BCG vaccination induced acceleration of epigenetic aging. This age-dependent divergence may be mediated, at least in part, by induction of CD38: BCG upregulates CD38 expression in hematopoietic stem cells (HSCs), which promotes HSC proliferation in youth, but contributes to HSC depletion and dysfunction in aged organisms. Together, these findings highlight the importance of comprehensively characterizing the effect of BCG vaccination, both as a model for probing the biology of aging and in the context of renewed interest in the heterologous benefits of vaccination, particularly in older populations.

Introduction

Bacillus Calmette-Guérin (BCG) remains the only available vaccine against tuberculosis. Although the protection it provides against pulmonary tuberculosis is suboptimal[1, 2], BCG is an effective vaccine against disseminated forms of the disease, especially in children. Subsequently, BCG vaccination is still one of the most widely administered vaccines worldwide. In addition to its specific antimycobacterial effects, BCG has also been shown to provide heterologous protection against unrelated non-mycobacterial infections and to reduce all-cause morbidity and mortality[3, 4]. It has been hypothesized that these heterologous effects of BCG are mediated at least partially by long-term enhancement of innate host defense mechanisms, a *de facto* innate immune memory termed ‘trained immunity’[5]. In addition, BCG vaccination was recently shown to reduce the concentrations of various inflammatory markers in the circulation [6], which was more pronounced in men than in women [7]. The training of the innate immune system by BCG is mediated through metabolic rewiring as well as epigenetic modifications in innate immune cells and their progenitors in the bone marrow [8].

One of the epigenetic layers that regulates gene expression is DNA methylation. BCG has already been shown to affect DNA methylation patterns of peripheral blood mononuclear cells (PBMCs) in adults and of monocytes in neonates[9, 10]. Interestingly, the process of aging is also tightly linked to changes in DNA methylation, leading to the hypothesis that epigenetic loss-of-information is an important mechanism of aging in humans and other vertebrates[11]. Based on the strong association between epigenetic modifications and age, extremely accurate age estimators based on DNA methylation could be constructed. Interestingly, among the various processes that promote cellular aging, recurrent or

severe infections have been suggested to be one of the most important[12]. It is not known whether vaccination can also impact the aging process, either directly or indirectly through the prevention of infections.

Because BCG has been shown to improve immune responsiveness[13] and down-regulate systemic inflammation[6, 7], both immune processes associated with aging of the immune system, we hypothesized that BCG vaccination may influence the process of aging of the immune system. Recently, a single-cell aging clock showed that $CD8^+$T cells exhibit diverse age-related alterations among individuals in response to BCG vaccination[14]. In the present study, we investigated the potential effect of BCG vaccination on epigenetic age scores. Several epigenetic age scores have been demonstrated to be strongly associated with lifespan (Horvath, Hannum) or health span (GrimAge, PhenoAge)[15]. To investigate our hypothesis, we analyzed two cohorts of BCG vaccinated individuals: (1) 284 young adults from the 300BCG study[7], with blood samples collected just before (baseline), two weeks, and three months after BCG vaccination; and (2) 117 elderly from the BCG-DENMARK-SENIOR trial[16], with samples obtained at baseline and one year post-vaccination. Genome-wide DNA methylation profiling was performed across all time points to assess how vaccination, host factors, and immune responses influence epigenetic aging.

Results

Study design and populations

To investigate the impact of BCG vaccination on epigenetic aging clocks, we included two distinct cohorts representing different age groups: the 300BCG study (a cohort of mostly young individuals) and the BCG-DENMARK-SENIOR study (a cohort of individuals above 65 years of age). The 300BCG cohort comprised 303 healthy volunteers who received BCG vaccination [7]. Blood samples were collected at baseline (T0), 14 days post-vaccination (T14), and three months post-vaccination (T3m). Genome-wide DNA methylation profiling, inflammatory protein quantification, and ex vivo cytokine response measurements were performed (Fig. 1). After quality control (see Methods), 284 participants (126 males, 158 females; mean age: 25.8 years, range: 18–71) were retained for downstream analyses (Fig. 1). The BCG -DENMARK-SENIOR cohort was based on a Danish randomized trial in which healthy individuals above the age of 65 were randomized to BCG or placebo and followed for 12 months[16]. Blood samples were obtained at baseline and 1-year post-vaccination. A sub-cohort of 121 elderly male volunteers who underwent BCG vaccination was randomly selected from this population for genome-wide DNA methylation analysis. After quality control, 117 subjects (mean age: 71.8 years, range: 65–90) were included in subsequent analyses (Fig. 1).

Sex-biased effect of BCG vaccination on epigenetic aging clocks

We first applied four epigenetic age (EA) models on data from the 300BCG study at the different time points, and we compared the performance of different models by the association between chronological and predicted age. Among the four models, the Horvath clock showed the best performance with the highest correlation coefficient and the lowest root mean square error (RMSE)(R = 0.86–0.88, RMSE = 5.61–6.40, Table S1; Fig. 2A) and was therefore used for downstream analyses. We next assessed changes in the epigenetic age acceleration (EAA, defined as the difference between predicted and chronological age) following BCG vaccination. A modest but significant reduction in EAA between baseline and three months post-vaccination was observed for the Horvath clock, whereas no net baseline-to–three-month change was detected for the Hannum, PhenoAge, or GrimAge clocks. (Fig. 2B). However, the Hannum and PhenoAge clocks showed transient changes at intermediate time points, with an increase at day 14 followed by a decrease by three months. (Fig. 2B). We also examined Intrinsic epigenetic age acceleration (IEAA), a measure of Horvath age acceleration adjusted for estimated blood cell composition, which showed a similar pattern to Horvath EAA, suggesting that estimated blood cell composition did not materially affect the results.

As our previous studies reported sex-dependent differences in DNA methylation changes to BCG vaccination [17], we next evaluated EAA separately in males and females. Interestingly, we identified a significant decrease in EAA after three months in men, but not in women (Fig. 3B, one-tailed paired t-test, P = 0.011 in all samples, P = 0.002 in males, P = 0.282 in females), with a mean decrease of epigenetic age of 1.58 years in men. This change already occurred 14 days post BCG vaccination, at which time we also identified a moderate change in men (mean decrease of 0.97 years), but not in women (Fig. 3A, one-tailed paired t-test, P = 0.807 in all samples, P = 0.047 in males, P = 0.998 in females).

To assess whether the BCG-induced change in EAA differed between sexes, we tested for a sex interaction using a model of EAA change from baseline to three months. The time × sex interaction did not reach conventional statistical significance (P = 0.08), indicating that while the reduction in EAA was predominantly observed in men, evidence for a statistically significant sex interaction was suggestive but not conclusive.

In line with earlier literature[18], baseline EAA differed significantly between males and females, with females exhibiting a significantly younger epigenetic age compared to males. Notably, this baseline difference was no longer observed following BCG vaccination (Figure S1). To explore potential contributors to baseline EAA, we examined associations with baseline characteristics. BMI showed no significant association with baseline EAA in either sex (Table S2). Among the five sex hormones measured, only hydroxyprogesterone (OHP) was positively correlated with baseline EAA in females (Table S2, Figure S1), which may partially contribute to the observed sex difference in baseline EAA. However, neither BMI nor sex hormone levels were associated with changes in EAA after BCG vaccination in either sex, suggesting that the BCG-induced EAA changes are independent of hormone concentrations. Given that BCG-induced DNA methylation changes have been linked to ex vivo cytokine responses, we further tested whether EAA changes were associated with cytokine responses. No significant associations were found in either sex (Table S2).

Decrease of epigenetic age after BCG vaccination is associated with activation of distinct immunological pathways

To further investigate the functional consequences of EAA changes in males, we compared single-cell transcriptomes at baseline and three months after BCG vaccination(T3m) in male participants whose EAA decreased at T3m versus those without a decrease. Individuals with decreased EAA showed enhanced transcriptional activation, particularly in $CD4^+$ and $CD8^+$ T cells, as indicated by a greater number of upregulated genes (Figure S2). In contrast, individuals without a decrease in EAA displayed a relative enrichment of downregulated genes in monocytes and NK cells. Although these patterns varied across cell types and conditions, they point to distinct immune response profiles associated with EAA changes.

Upon LPS stimulation, used to mimic secondary infection, both groups showed increased transcriptional responses; However, the cellular distribution of differentially expressed (DE) genes differed between groups, with monocytes contributing a larger share of DE genes in the decreased EAA group, whereas $CD8^+$ T cells and NK cells were more prominent in the non-decreased group. Pathway enrichment analysis of LPS-induced upregulated genes further revealed enrichment of pathways related to host–pathogen interaction, apoptosis, and tight junctions in $CD4^+$ T cells, as well as NK cell-mediated cytotoxicity in $CD8^+$ T cells.

BCG vaccination decreases the inflammation age clock

In a previous study, we showed that BCG vaccination leads to a reduction in circulating inflammatory markers in a sex-dependent manner[7]. Because systemic inflammation is a well-established hallmark of aging, we generated an inflammation age clock based on circulating inflammatory protein concentrations. The inflammation-age clock was initially built on a cohort of 500 healthy volunteers (500FG cohort) who were not vaccinated with BCG and subsequently showed good performance in the test data (the present 300BCG cohort). The correlation coefficient between chronological and predicted age ranged from 0.76 to 0.79 (for each time point, Fig. 4). We observed an overall decrease in inflammation-age at three months after BCG vaccination (one-tailed paired t-test, $P = 0.015$ in all samples, mean decrease of 0.42 years), but not for T14. A similar decreasing trend was observed in both men and women, although it was not significant in men (mean decrease of 0.39 years in males, $P = 0.091$, Fig. 4, Table S3).

BCG vaccination increases epigenetic age acceleration in an older adult population

We subsequently assessed whether the effects of BCG vaccination on epigenetic clocks vary depending on the age of the individuals. The 300BCG cohort primarily consisted of young adults, with only 18 participants over the age of 50, among whom 9 were male. We first conducted a stratified analysis

based on chronological age and found that the decrease in EAA was observed exclusively in the younger male population (Figure S3A). In contrast, an opposite trend was seen in the older group, although the small sample size limited the statistical significance of this finding.

To further evaluate the impact of BCG vaccination on epigenetic aging in an older population, we analyzed data from an independent cohort, BCG-DENMARK-SENIOR. From this cohort, we analyzed samples from a random selection of 117 male participants. Their mean age was 71.8 years (range: 65–90.4) at the time of BCG vaccination. Consistent with findings from the 300BCG cohort, the Horvath clock demonstrated the best performance in the BCG- Denmark-SENIOR cohort, with the highest correlation coefficients (R = 0.45–0.52) and RMSE values (11.95–14.92), followed by PhenoAge (R = 0.40–0.42, RMSE = 7.89 − 8.22) (Table S4, Fig. 5). However, the overall performance of epigenetic clocks in the older population was lower compared to that in the younger cohort. In contrast to the findings in young adults, EAA significantly increased after BCG vaccination in older men, with a mean increase of 4.30 years of the Horvath clock (paired t-test, $P = 1.01 \cdot 10^{-12}$). This effect was observed irrespective of chronic disease status (Figure S3B). These findings suggest that, unlike in young individuals, BCG vaccination may be associated with accelerated epigenetic aging in older males.

BCG vaccination effects on epigenetic age acceleration may be mediated through modulation of CD38 expression

BCG-induced effects on innate immune cells have been shown to be exerted at the level of hematopoietic stem and progenitor cells (HSPCs), and HSPC senescence is probably the most important cellular process that underlies immune aging[19]. CD38 expression in HSPCs has been previously shown to have opposite effects on aging in young vs old organisms[20]. Based on this, we examined the effects of BCG vaccination on CD38 expression. Interestingly, BCG administration led to an increased expression of CD38 in HSCs three months after vaccination (Fig. 6A). To further explore the potential role of CD38 in EAA, we analyzed published GWAS summary statistics of EAA[21] and identified CD38-associated SNPs, among which rs6849988 was significantly associated with EAA (P = 0.002) (Fig. 6B). Finally, we assessed the correlation between CD38 expression and EAA in the peripheral blood samples from the Senior Individual (SI) cohort, a cohort focusing on aged individuals, (Roesner et al., 2024), Consistent with the opposing effects of CD38 across age groups, CD38 expression was inversely correlated with the EAA score in young individuals, but positively associated in the elderly (Fig. 6C). These findings suggest that the impact of CD38 expression on epigenetic aging is age-dependent and may contribute to the modulatory effects of BCG vaccination on EAA.

Discussion

BCG vaccination is known to exert important protective heterologous effects on infections, including non-mycobacterial [22], and an increasing number of studies suggest an impact on cancer[23], autoimmune diseases[24, 25], and neurodegenerative diseases[26, 27]. For many of these diseases, age is one of the main risk factors, which triggered us to investigate the impact of BCG vaccination on the

aging process as assessed by epigenetic age clocks. Interestingly, BCG vaccination exerted complex age- and sex-specific effects on epigenetic age clocks. In young individuals, BCG vaccination significantly decreased the EAA, especially in men. This was also associated with a decrease in an inflammatory age score as well. In contrast, BCG vaccination increased EAA in elderly men. This effect is likely to involve CD38-mediated mechanisms: BCG induces CD38 in HSCs, a factor shown earlier to promote HSC proliferation at a young age, but a decrease in HSCs in aged organisms[28].

Aging is one of the most important risk factors for infections, diabetes, neurodegeneration, cardiovascular diseases, and cancer[29]. These effects are believed to be mediated at least in part by changes associated with the aging immune system that can lead to both a process of low-grade chronic systemic inflammation ("*inflammaging*"), and a defective responsiveness of the immune system to pathogens and malignant processes ("*immunosenescence*"). While chronological age is a strong predictor of disease risk, increasing evidence indicates that the biological age of the organism, assessed through molecular and physiological biomarkers, may provide a better reflection of the aging process[30, 31]. Among such biomarkers, epigenetic age clocks are calculated based on changes in DNA methylation: loss of DNA methylation is believed to result in dysregulated gene expression and aging[32]. Several epigenetic age clocks have been developed, some predicting the lifespan (Hannum, Horvath clocks) and others predicting health span (GrimAge, PhenoAge) of an individual[33–35]. Interestingly, BCG vaccination exerts several important molecular and physiological effects that may affect the process of aging of the immune system: BCG vaccination is associated with long-term changes in DNA methylation in immune cells[9, 10, 17], it improves cell responsiveness[13, 36], and it reduces inflammation[7]. That prompted us to investigate its effects on epigenetic age clocks.

We investigated these effects in two independent human cohorts: one recruited in the Netherlands (300-BCG) and one in Denmark (BCG-DENMARK-SENIOR). The 300-BCG cohort consisted of approximately 300 mostly young individuals (80% under the age of 30 years): in this cohort, we observed a significant decrease in epigenetic age acceleration, especially in men, and less strongly in women. The cause of this difference is not known, but it is also likely be due to the intrinsic higher EAA in men compared with women[37, 38], also observed in our study. An effect of BCG vaccination on slowing the process of aging in young individuals was also supported by the BCG-induced reduction of an inflammatory age score based on 92 inflammatory mediators. As both epigenetic and inflammatory age clocks are biochemical readouts, this observation needs to be complemented in the future by larger studies that may demonstrate the consequences of these effects at the clinical level. Interestingly, the impact of BCG vaccination on epigenetic age acceleration was associated with different transcriptional effects in immune cells: individuals who reacted with decreased EAA after BCG vaccination also displayed an enhanced transcriptional response in CD4 + and CD8 + lymphocytes, while those in whom no change in EAA was observed showed downregulation of transcriptional processes in NK cells and monocytes. These findings demonstrate the relevance of the DNA methylation response post-BCG on activation of various immune cell populations.

Probably the most intriguing observation is that the effects of BCG vaccination on epigenetic age clocks seem to be in the opposite direction in elderly individuals. As the effects of BCG vaccination in the young volunteers were observed only in men, we focused on assessing changes in DNA methylation after vaccination in men in a cohort of elderly individuals (BCG-DENMARK-SENIOR). BCG vaccination significantly and consistently increased EAA calculated on the basis of Horvath, Hannum and PhenoAge clocks (the exception being GrimAge clock, for which no effect was observed). However, the observed positive EAA in older individuals should be interpreted cautiously, as the Horvath clock showed substantially reduced performance in this age group compared to EAA in this population, and the positive EAA may reflect reduced clock performance rather than a true biological effect.

Complex effects of BCG vaccination have also been shown on other hallmarks of aging such as telomere attrition. A recent study has reported increased shortening of telomeres after BCG vaccination in men, but not in women, an effect dependent on testosterone concentrations[39]. Furthermore, a recent single-cell transcriptomic analysis reported that BCG vaccination induced both immune “rejuvenation” and “acceleration” phenotypes across individuals, indicating substantial inter-individual variability in biological aging responses following vaccination. Together with the present findings, these results suggest that BCG may influence multiple dimensions of biological aging, including epigenetic, telomeric, and .-transcriptomic markers, which may not change in parallel. Collectively, this implies that the effects of BCG vaccination on the aging process may differ with the age and sex of the individual, as well as the baseline immune state, all factors that needs to be taken into consideration in future clinical studies.

An important question that arises concerns the molecular mechanisms responsible for the potential differential effects of BCG vaccination in young or old individuals. We propose that at least part of this difference may be due to the capacity of BCG vaccination to increase the expression of CD38 in HSCs of healthy volunteers (Fig. 6A). CD38 is an NAD+-dependent metabolic enzyme that has been recently shown to promote HSC proliferation by inducing mitochondrial Ca2 + influx and mitochondrial metabolism in young mice[28]. In contrast, an increase in CD38 expression during aging is a driver of HSC deterioration due to dysregulated NAD+ metabolism and compromised mitochondrial stress management[28]. Polymorphisms in CD38 significantly influence EAA (Fig. 6B), although the mechanism of this effect is not known. Furthermore, expression of CD38 was associated with a decrease in EAA in individuals younger than 30 years, but with an increased EAA in older individuals above 70 years of age, confirming its age-dependent effect (Fig. 6C). While CD38 may represent one mechanistic link, other explanations are also plausible, including age-related differences in the baseline epigenetic landscape, immune cell composition, and vaccination history.

Our study also has some limitations. First, the number of individuals studied is relatively small, and larger validation studies are warranted. Second, the follow-up period was short, precluding assessment of clinical outcomes. Third, the cohorts consist mainly of individuals of European ancestry, and future studies should investigate the effects of BCG vaccination in more diverse populations. Fourth, the study populations differed with respect to both BCG strain (BCG-Bulgaria vs. BCG-Danish) and vaccination history, with the Danish cohort largely receiving revaccination and the 300BCG cohort undergoing

primary vaccination. As both vaccine strain and prior exposure have been reported to influence BCG-induced responses, their contribution to the observed differences between cohorts cannot be excluded. Finally, further mechanistic studies are needed to better understand the effects of BCG vaccination on aging

In conclusion, we observed complex age- and sex-specific effects of BCG vaccination on epigenetic age clocks, with decreased EAA after BCG vaccination in young men, but an increased EAA post-vaccination in elderly individuals. The relevance of these findings is apparent at several levels. First, understanding the full effects of BCG vaccination is crucial in the face of a growing interest in heterologous effects of vaccines in general, and BCG vaccination in particular, including in the elderly population. Second, these data argue that BCG vaccination may be used as a useful tool to investigate the aging process. Finally, our study indicates a need for extending these investigations to other vaccines and infections as well, as they may also exert similar (or different) effects on the aging process. This would be increasingly important to understand, considering the increasing rate of infections and vaccinations to which elderly individuals are exposed.

Methods

Human cohorts

300BCG cohort

To study the immunological effects of BCG vaccination, healthy adult volunteers of Western European ancestry were included in the 300BCG study between April 2017 and June 2018, as previously described[7,40]. After written informed consent was obtained, a standard dose of 0.1 mL BCG (BCG-Bulgaria, InterVax) was administered intradermally in the left upper arm. EDTA blood was collected before, as well as two weeks and three months after vaccination. Volunteers were not eligible for participation in case of systemic medication use other than oral contraceptives or acetaminophen, previous BCG vaccination, use of antibiotics three months before inclusion, any vaccination three months before or during the study, any febrile illness four weeks before participation, or a medical history of immunodeficiency. Participants were also not eligible if they recalled a history of tuberculosis or had lived in a tuberculosis-endemic setting. After BCG administration, the study participants received an extensive online questionnaire about lifestyle, diet, and disease history. The study was approved by the Arnhem-Nijmegen Medical Ethical Committee (NL58553.091.16) and performed in accordance with the Declaration of Helsinki.

BCG- DENMARK-SENIOR study

Danish citizens aged 65 years or older, with access to secure electronic mail from the authorities, were eligible. Exclusion criteria were known contraindications to BCG vaccination and acute infection at the time of vaccination. Participation was based on voluntary enrolment following oral and written consent. Recruitment took place from September 2020 to December 2021 in the city of Odense. Participants were

randomized 1:1 to receive either a standard dose BCG vaccination (BCG strain 1331, AJ Vaccines, Denmark) or placebo (saline). Randomization was stratified by sex and age group (< /≥75 years of age). Participants were followed for 12 months post-randomization. Blood samples were obtained at baseline and 12 months post-randomization. Previous BCG vaccination, mostly in childhood, was reported by 98% of participants. The study was approved by the Ethics Committee of the Region of Southern Denmark (approval number S-20200134C) and the Danish Medicines Agency (approval number 2020081033) according to the principles of the Helsinki Declaration and Good Clinical Practice (GCP) guidelines. The study was monitored by the GCP unit at Odense University Hospital. For the present study, a sub-cohort of male participants aged 65-84 years, randomized to BCG was randomly selected among participants with paired samples

DNA methylation measurement and quality control

DNA was extracted from whole blood using a QIAamp blood kit (Qiagen Benelux BV, Venlo, the Netherlands) kit, and the concentration was measured using a NanoDrop spectrophotometer at 260 nm. Genome-wide DNA methylation was assessed using Infinium© MethylationEPIC array (~850,000 CpG sites). Raw IDAT files generated by the HiScanSQ scanner were processed in R (version 4.0) using the minfi package (Bioconductor)[41]. Samples with low call rates (n = 4) or sex mismatch (n = 1) were excluded. Sample identity was further verified by inspecting correlations of beta values at SNP probes; mislabeled samples were relabeled if the correlation exceeded 0.7. Quality control included the removal of probes with detection P-values > 0.01, cross-reactive probes, polymorphic CpGs[42,43], and probes located on sex chromosomes. Stratified quantile normalization was then applied to the filtered dataset[44].

DNA methylation age calculation

DNA methylation age (DNAm age) were generated by DNA Methylation Age Calculator (https://dnamage.genetics.ucla.edu/home)[33], and four common DNAm age clocks were generated: Horvath clock, Hannum clock, GrimAge clock, and PhenoAge clock. EAA was defined as residual from regressing DNAm age on chronological age.

Inflammation age calculation

The inflammation age model was initially developed using data from the 500FG cohort, which has been described in detail previously[45]. Inflammatory markers were measured in EDTA plasma using the commercially available Olink® Inflammation Panel (Olink Proteomics AB), which quantifies 92 inflammatory proteins via the Proximity Extension Assay (PEA) technology on the Proseek Multiplex platform. Prediction of the chronological age was performed by training an Elastic Net model. The dataset was randomly split into a training set (70%) and a test set (30%). Feature selection was performed on the training set using 10-fold cross-validation, repeated five times to ensure robustness. Prediction performance was evaluated on the test set by calculating the Pearson correlation coefficient and root mean squared error (RMSE) between the predicted and actual chronological ages. This entire

procedure was repeated 100 times to minimize the impact of random variation. The trained models were then applied to the 300BCG cohort using the measured inflammatory protein levels as input. The average predicted age across the 100 models was taken as the inflammation age for each individual in the 300BCG cohort.

CD38 expression in HSCs

CD38 expression was assessed in hematopoietic stem cells from 20 BCG-naive volunteers, both male and female, aged between 18 and 50 years, who were administered either placebo (n=5) or BCG vaccine (n=15), as described in Cirovic et al.[46]. These participants were healthy, with no active infections, no signs of inflammation, and a negative Quantiferon-TB Gold test result. The trial was approved by the Arnhem-Nijmegen Ethical Committee (approval number NL55825.091.15). Single-cell sequencing was performed as described by Sun et al [47], and CD38 expression data were evaluated in the hematopoietic stem cells.

CD38 locus and expression analyses

To assess CD38 polymorphisms in relation to EAA, we used the European-ancestry GWAS summary statistics from McCartney et al[48]. Variants within the CD38 locus (±250 kb) were extracted and subjected to disequilibrium (LD) in PLINK v1.9 ($r^2 < 0.1$, 250 kb window) using the HapMap EUR reference panel. Independent lead SNPs were identified, and multiple testing correction (using the Bonferroni method) was applied based on the number of clumped variants. Locus-specific association plots were generated to visualize CD38 SNPs associated with EAA (Horvath's clock).

Log2-transformed CD38 gene expression values were obtained from the RESIST Senior Individuals (SI) cohort[49], following normalization using the trimmed mean of M-values (TMM) method implemented in the edgeR package. These values were analyzed for association with epigenetic age acceleration (EAA; Horvath's clock), obtained from https://dnamage.clockfoundation.org/, using linear regression (lm function in R). Analyses were conducted separately for young adults (age < 30 years, n = 9) and older adults (age > 70 years, n = 104). P-values were adjusted for multiple testing using the Benjamini–Hochberg false discovery rate (FDR) method.

Single-cell RNA sequencing

Peripheral blood mononuclear cells (PBMCs) were isolated from participants before and three months after BCG vaccination. Following ex vivo stimulation with either RPMI medium or LPS (10 ng/mL), cells were processed for single-cell RNA sequencing using the 10x Genomics Chromium Next GEM Single Cell 3' Library & Gel Bead Kit v3.1 and Chip G. Libraries were pooled (32 in total, combining cells from 3–5 donors per pool) and sequenced on an Illumina NovaSeq 6000 platform (28 bp R1, 90 bp R2). Raw sequencing data were processed using the CellRanger v3.1.0 pipeline and mapped to the GRCh38 reference genome. Cell demultiplexing was performed using Souporcell v2.0[50], achieving ~92% cell recovery. Low-quality cells (mitochondrial gene content > 25% or < 100 /> 5000 detected genes) and

lowly expressed genes (expressed in < 5 cells) were excluded, yielding ~200,000 high-quality cells and 21,975 genes for downstream analyses using the Seurat (v4.0.0)[51] package of R (v4.0.2).

Statistical analyses

All computational analyses were conducted using R version 4.0. Paired t-tests were used to compare DNAm age between time points, and one-tailed p-values were calculated to assess whether DNAm age significantly increased after BCG vaccination. Linear regression models were applied to examine associations between baseline characteristics (BMI and hormone levels) and both baseline EAA and changes in EAA. Associations between EAA changes and ex vivo cytokine responses were also assessed using linear regression. All p-values from linear regression analyses were adjusted for multiple testing using the false discovery rate (FDR) method.

Declarations

Declaration of interests

MGN is the scientific founder of Biotrip, Salvina, TTxD and Lemba TX. YL and MGN are scientific founders of Numenor Tx.

Competing Interests

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Author Contribution

Author C ContributionsC.-J.X. and M.G.N. conceived and supervised the study. C.Q. designed the analytical framework, performed the computational and statistical analyses, interpreted the results, and drafted the manuscript. Y.Z., M.B., Z.L., W.L., A.N., and V.K. contributed to data processing, statistical analyses, and interpretation. S.M., C.d.B., V.K., V.P.M., M.J., L.A.B.J., and M.G.N. designed, coordinated, and conducted the BCG vaccination studies and oversaw sample collection. S.S. and L.B.B. contributed to data generation, analysis, and interpretation. F.S.-B., A.M.R.M., and C.S.B. contributed to the design of the Danish BCG study, sample collection, and interpretation of the findings. L.M.R., J.H., and T.W. contributed to data generation, interpretation, and critical discussion of the findings. Y.L. contributed to study design, data interpretation, and supervision. C.-J.X. supervised the computational and statistical work and, together with M.G.N., directed the overall project. All authors critically revised the manuscript, approved the final version, and agreed to be accountable for all aspects of the work.

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Data availability

300BCG DNA methylation data have been deposited in the European Genome-phenome Archive (EGA), hosted by the EBI and CRG, under accession number EGAS00001007498. BCG-DENMARK-SENIOR data have also been deposited under accession number EGAS00001008446 (to be released)

Code availability

Code generated to process the data is freely available on GitHub (https://github.com/CiiM-Bioinformatics-group/300BCG_epigenetic_aging).

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Figures

Figure 1

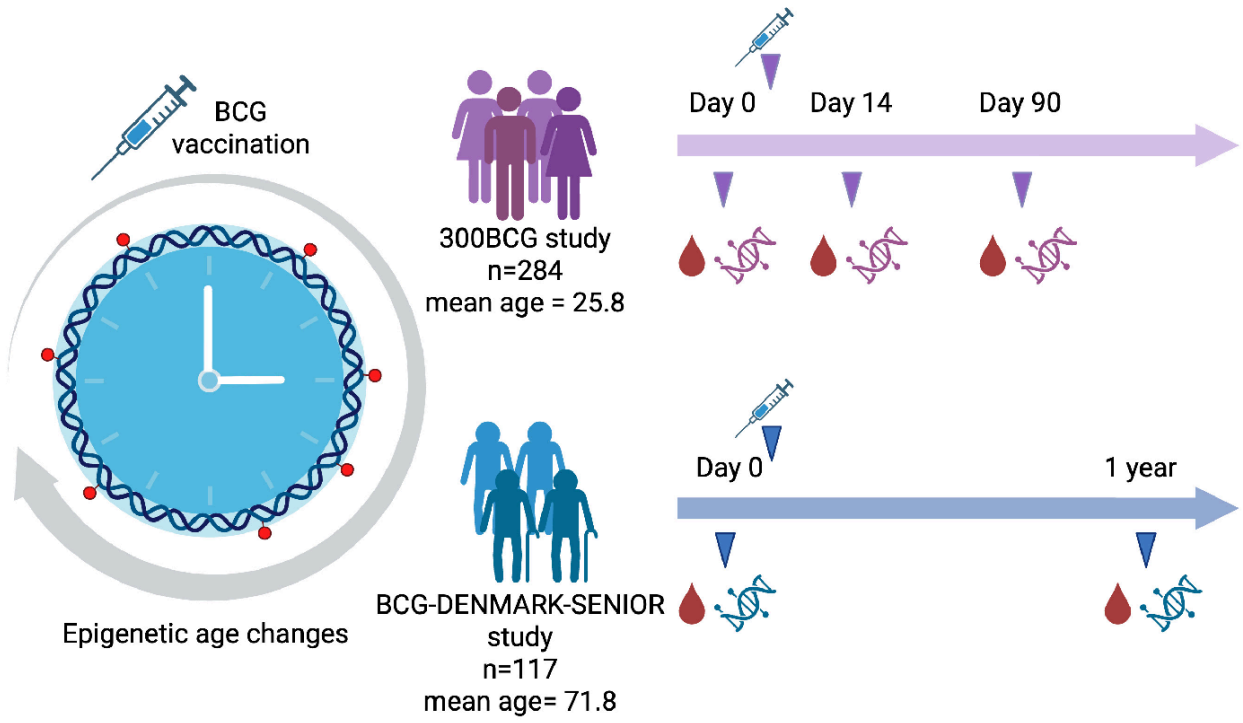


Figure 1

Study design

Participants in the 300BCG study (n = 284, mean age 25.8 years) and the BCG-DENMARK-SENIOR study (n = 117, mean age 71.8 years) received BCG vaccination at day 0. Blood samples were collected at baseline and follow-up time points (day 14 and day 90 in 300BCG; one year in BCG-DENMARK-SENIOR) for DNA methylation profiling and assessment of epigenetic age changes.

Figure 2.

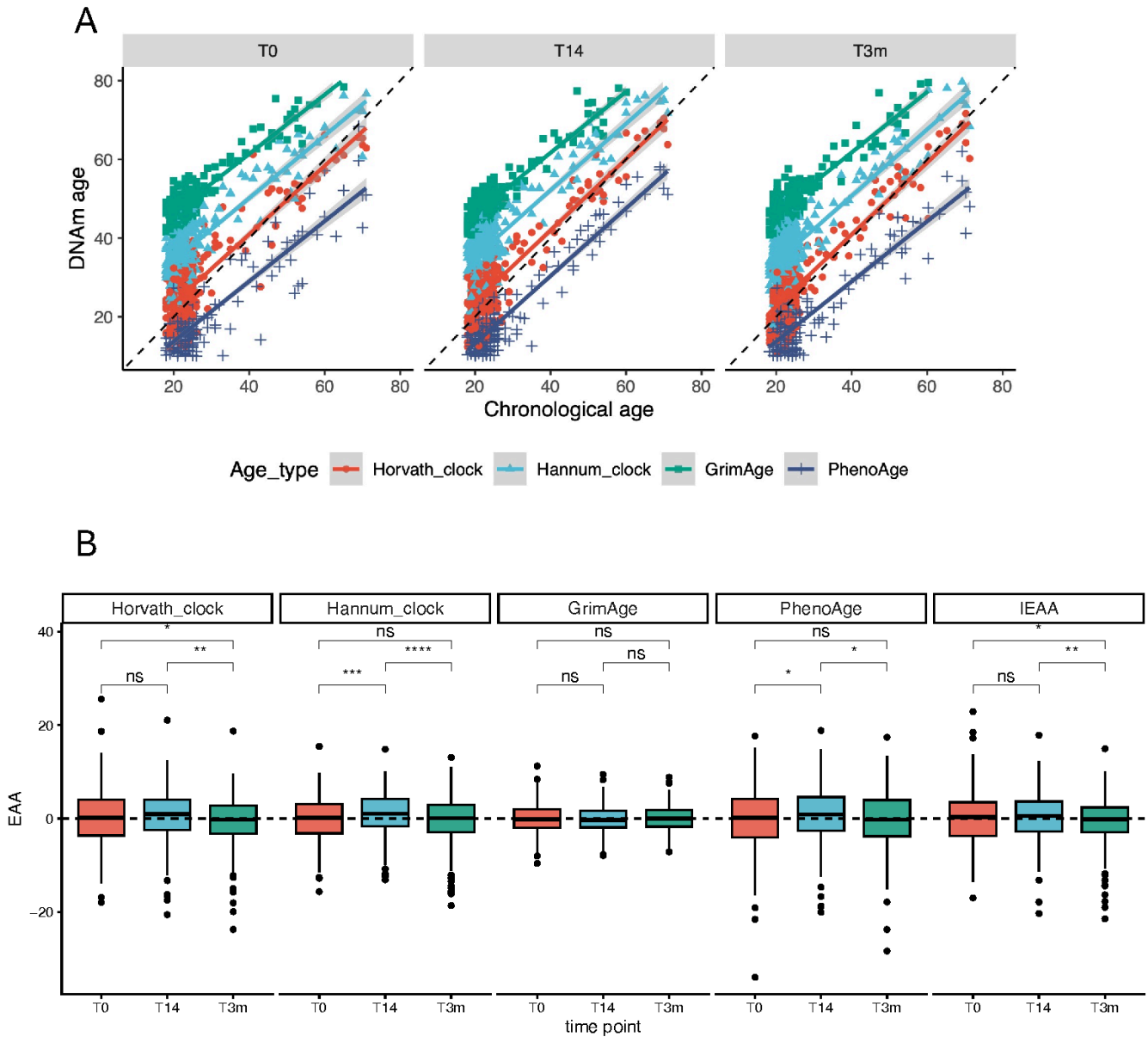


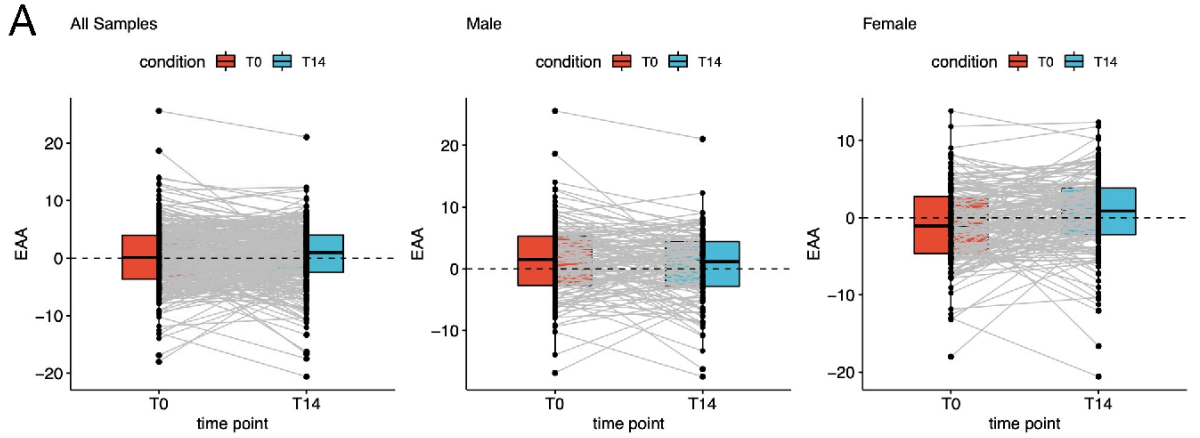
Figure 2

Association between epigenetic age clocks and chronological age and time-dependent effects of BCG vaccination

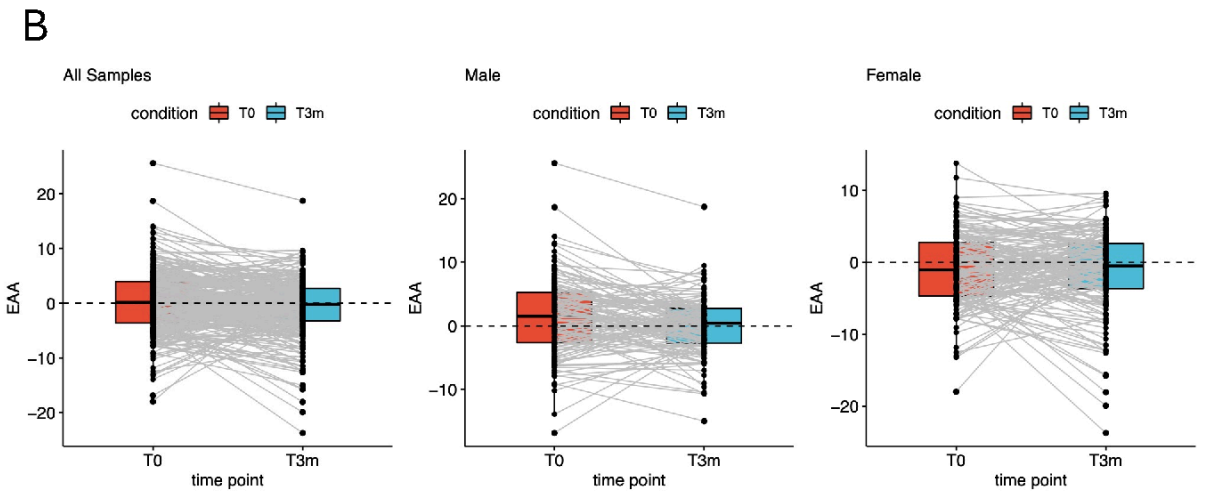
(A) DNA methylation–based age estimates derived from the Horvath, Hannum, PhenoAge, and GrimAge clocks plotted against chronological age at baseline (T0), 14 days (T14), and three months (T3m) after BCG vaccination. The dashed line represents the line of identity (DNA age = chronological age). All

epigenetic clocks showed strong correlations with chronological age across all time points.
(B) Epigenetic age acceleration (EAA) and intrinsic epigenetic age acceleration (IEAA) for each clock at T0, T14, and T3m in the entire cohort. Boxplots show the median and interquartile range, with individual data points overlaid. Modest but significant changes in EAA were observed at specific time points for selected epigenetic clocks, while no consistent effect was detected across all clocks. Statistical significance is indicated as ns (not significant), *P < 0.05, **P < 0.01, ***P < 0.001, and ****P < 0.0001.

Figure 3.



Comparison	DNAm-age T14– T0 (all samples)	DNAm-age T14– T0 (Male)	DNAm-age T14– T0 (Female)
P value	0.807	**0.047**	0.998
Mean of difference	0.315	-0.971	1.329



Comparison	T3m– T0 (all samples)	T3m– T0 (Male)	T3m– T0 (Female)
P value	**0.011**	**0.002**	0.282
Mean of difference	-0.859	-1.579	-0.293

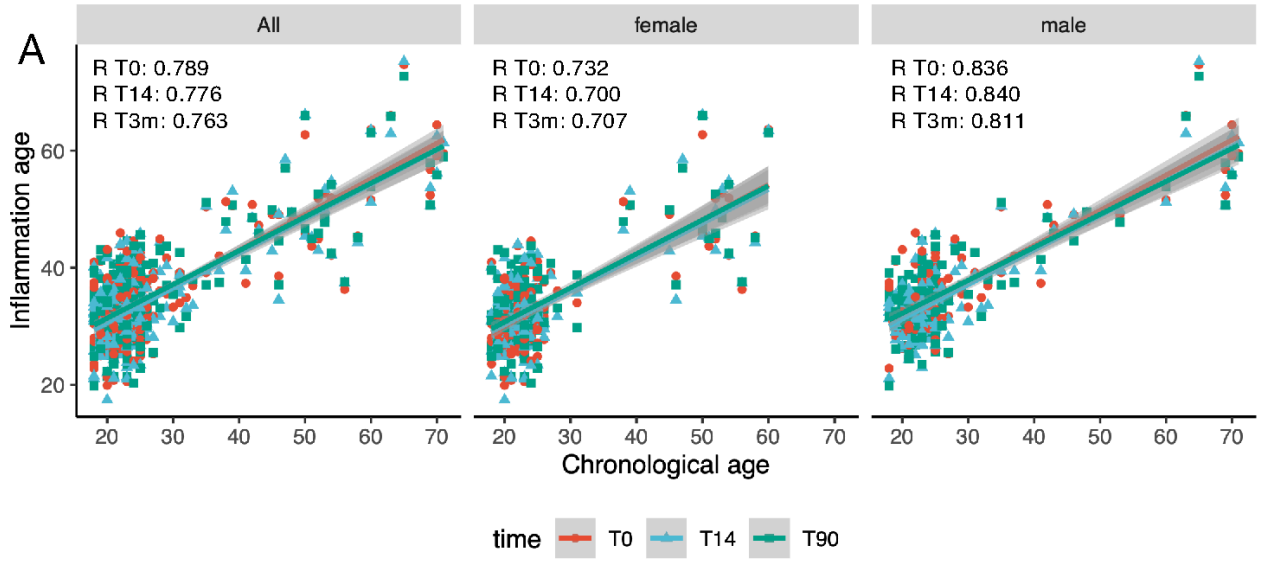
Figure 3

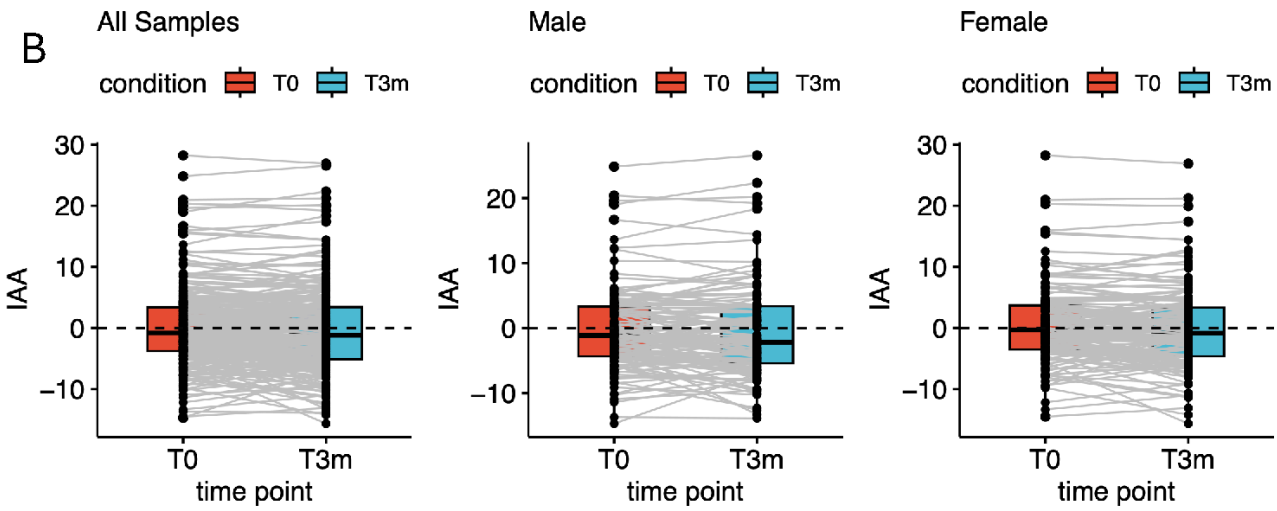
BCG vaccination induces a sex-specific reduction in epigenetic age acceleration

(A)Epigenetic age acceleration (EAA) at baseline (T0) and 14 days (T14) after BCG vaccination in all participants and stratified by sex. Paired comparisons show a significant reduction in EAA at T14 in men, but not in women. B) Epigenetic age acceleration at baseline (T0) and three months (T3m) after BCG vaccination. A significant decrease in EAA was observed at T3m in men, with a smaller but significant effect in the combined cohort, whereas no significant change was detected in women. Boxplots indicate median and interquartile range, with individual paired measurements connected by grey lines. P values and mean differences (ΔEAA relative to baseline) are shown below each panel.

Comparison is performed by paired one-tailed t-test in all samples (left panel), male (middle panel) and female (right panel), respectively.

Figure 4.





Comparison	T3m – T0 (all samples)	T3m – T0 (Male)	T3m – T0 (Female)
P value	**0.015**	0.091	0.043
Mean of difference	-0.422	-0.386	-0.451

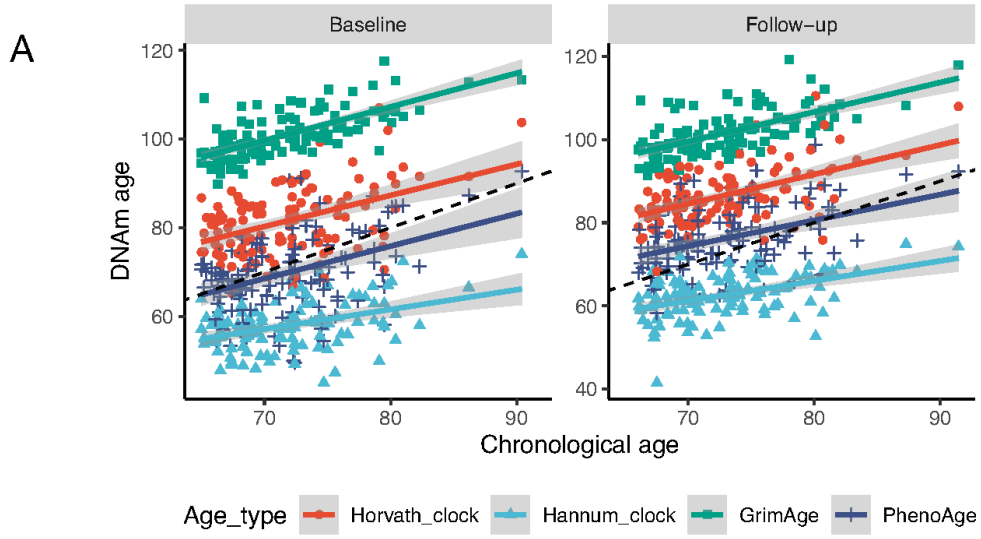
Figure 4

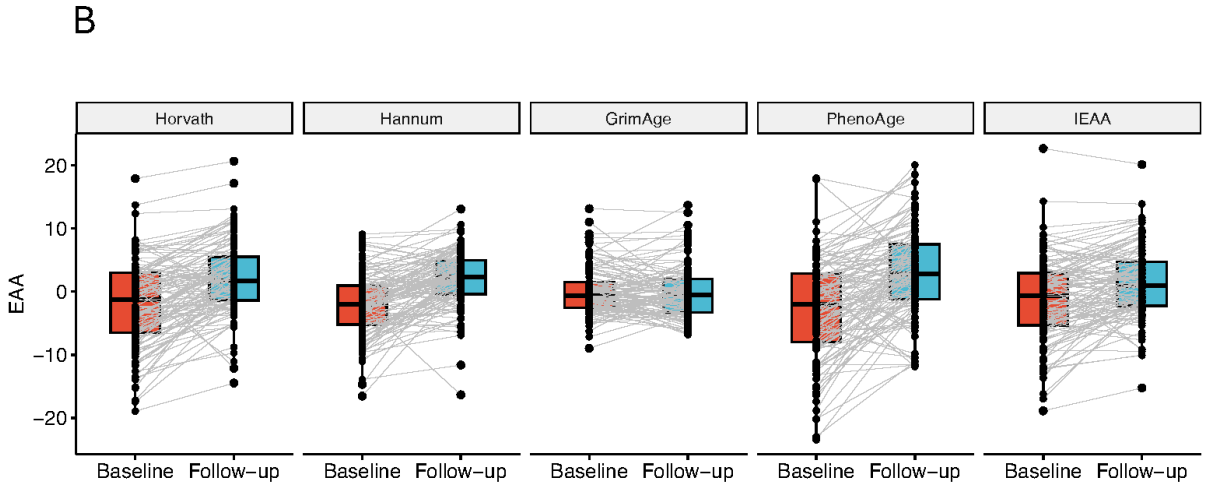
BCG vaccination decreases the inflammation age score

A. Inflammation age score built on 92 circulating inflammatory biomarkers. Correlation between chronological age and inflammation age is assessed by Pearson correlation test. B. Boxplots illustrating changes in inflammation age acceleration (IAA, as the difference between predicted and chronological

age) at month 3 after BCG vaccination comparing with baseline (300BCG cohort). Comparison is performed by paired one-tailed t-test in all samples (left panel), male (middle panel) and female (right panel), respectively.

Figure 5.





Age clock	Horvath clock	Hannum clock	GrimAge	PhenoAge	IEAA
P value	1.01e-12	2.59e-14	0.49	2.61e-12	1.70e-7
Mean difference (Baseline - Follow-up)	-4.30	-4.12	0.25	-5.54	-2.70

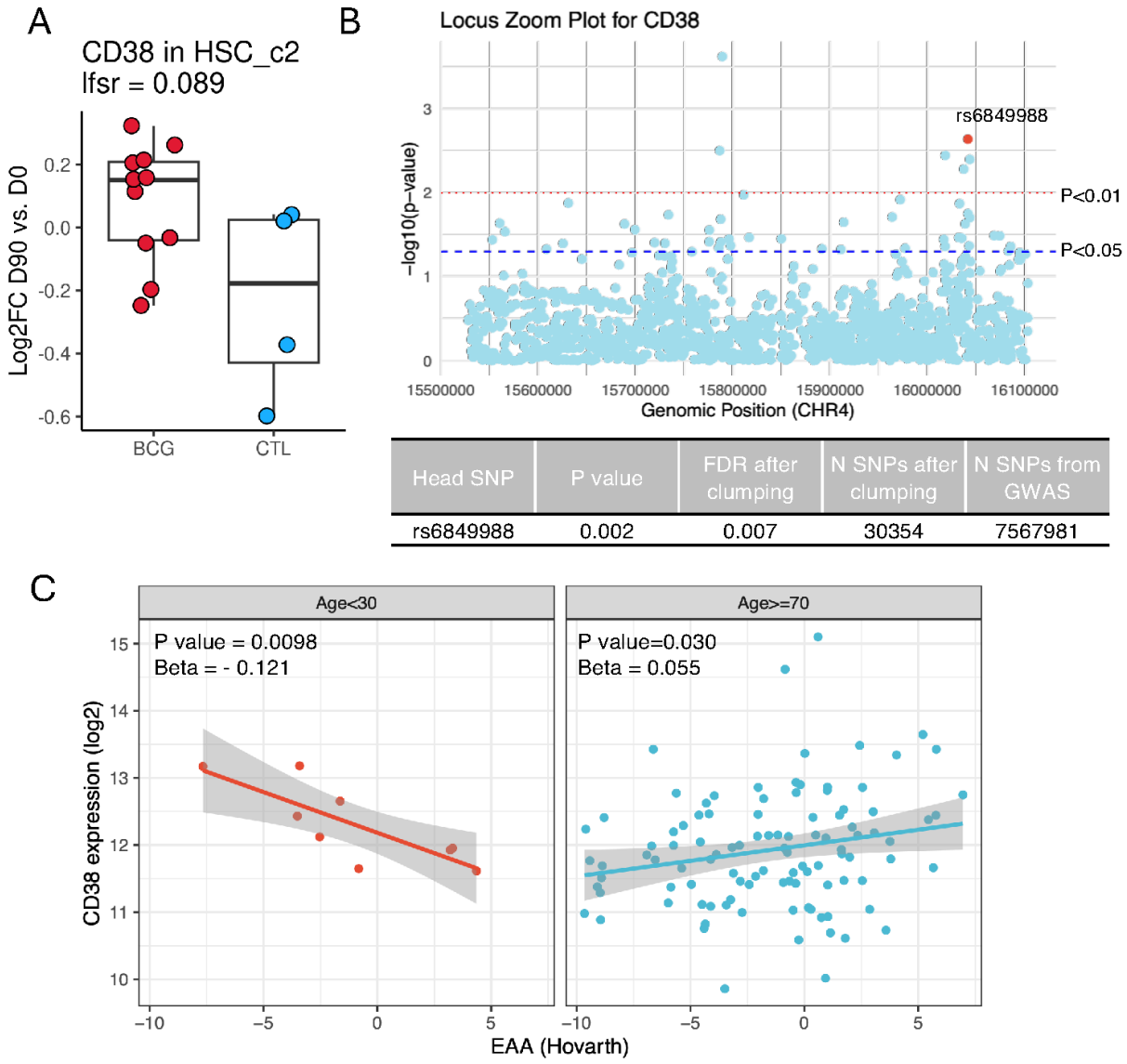
Figure 5

BCG increases epigenetic age acceleration (EAA) in a cohort of elderly men

A. Association between Horvath, Hannum, PhenoAge, and GrimAge scores and chronological age in the BCG-Denmark-SENIOR cohort.

B. Boxplots illustrating changes in epigenetic age acceleration (EAA, defined as the difference between predicted and chronological age) and intrinsic epigenetic age acceleration (IEAA) between baseline and 1-year follow-up after BCG vaccination. Statistical comparisons were performed using a paired t-test.

Figure 6



Head SNP	P value	FDR after clumping	N SNPs after clumping	N SNPs from GWAS
rs6849988	0.002	0.007	30354	7567981

Figure 6

BCG vaccination effects on epigenetic age acceleration modulated by effects on CD38 expression

A. BCG induces expression of CD38 in HSCs from healthy volunteers. B. CD38 SNPs influence EAA. C. CD38 expression in volunteers under the age of 30 years is associated with lower EAA. D. CD38 expression in volunteers over the age of 70 years is associated with higher EAA.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplementaryfigures4.9.pdf
- Supplementaltables12.10.xlsx