Tuberculin responses after BCG vaccination predict amyotrophic lateral sclerosis risk

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ABSTRACT

Background: T cell infiltration around dying motor neurons is a hallmark of amyotrophic lateral sclerosis (ALS). It is not known if this immune response represents a cause or a consequence of the disease. We aimed to establish whether individual variation in regulation of a T cell driven immune response is associated with long-term ALS risk.

Methods: Tuberculin skin test (TST) following BCG vaccination represents a standardized measure of a secondary T cell driven immune response. During a Norwegian tuberculosis screening program (1963–1975) Norwegian citizens born from 1910 to 1955 underwent TST. In those previously BCG vaccinated (median 7 years prior to TST), we related tuberculin skin tests to later ALS disease identified through validated Norwegian health registers. We fitted Cox proportional hazard models to investigate the association between tuberculin reactivity and ALS risk.

Results: Among 324,629 participants (52 % women) with median age 22 (IQR 10) years at tuberculosis screening, 496 (50 % women) later developed ALS. Hazard ratio for ALS was 0.74 (95% CI 0.57–0.95) for those who remained TST negative compared to those who mounted a positive TST. The association was strongest when time between BCG immunization and TST was short. The associations observed persisted for more than four decades after TST measurement.

Conclusions: Negative TST responses after BCG vaccination is associated with decreased long-term risk for ALS development, supporting a primary role for adaptive immunity in ALS development.

1. Introduction

Compelling evidence suggest that adaptive immune responses are involved in amyotrophic lateral sclerosis (ALS) (Beers and Appel, 2019), a fatal neurodegenerative disease of mainly motor neurons. Neuro-inflammation including T cell infiltration and activation of microglia and astrocytes in areas of motor neuron degeneration in the brain and spinal cord from ALS patients was early reported (Engelhardt et al., 1993; Kawamata et al., 1992; Troost et al., 1990). More recent studies have revealed that infiltration of CD4^{+} T cells is associated with microglia activation (Henkel et al., 2009). Clonally expanded CD4^{+} and CD8^{+} cells from the cerebrospinal fluid of ALS patients have an activated phenotype compatible with an antigen-driven response (Yazdani et al.,

2022). Patients with ALS have high levels of Th17 and Th1 cells and corresponding pro-inflammatory cytokines such as interferon gamma, and conversely decreased function and numbers of regulatory T cells (Treg) (Hu et al., 2017; Jin et al., 2020). High number of Tregs and a high ratio between activated and resting Tregs are associated with long survival (Sheean et al., 2018; Yazdani et al., 2022), whereas high levels of Th1/Th17 cells are associated with short survival (Yazdani et al., 2022).

Whereas it was previously believed that the healthy brain was secluded from the adaptive immune system, it is now established that adaptive immune cells support brain function and that they also may facilitate repair (Castellani et al., 2023). On the other hand, imbalanced immune responses may contribute to tissue damage. The target antigen

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of the immune response in ALS is not known (Ramachandran et al., 2023), and it is not known whether T cell infiltration around dying motor neurons and alterations in immune cell subsets in blood and cerebro spinal fluid (CSF) of ALS patients represent primary events in the disease process, or are merely epiphenomena. A primary role for adaptive immunity is suggested by epidemiological studies reporting increased ALS risk in persons diagnosed with autoimmune diseases (Turner et al., 2013). These associations are however strongest the last year before ALS diagnosis, particularly for autoimmune diseases that could mimic ALS (Cui et al., 2021). Misdiagnosis could therefore explain some of these associations.

Genetic data are also conflicting on whether immune responses are primary players in ALS development. A comprehensive study using summary statistics from large genome wide association studies and false discovery rate statistics, found robust genetic enrichment between ALS and celiac disease and multiple sclerosis and moderate enrichment between ALS and ulcerative colitis and type 1 diabetes (Li et al., 2021). A Mendelian randomization study did however not confirm these results (Alipour et al., 2022).

If T cells play a primary role, one would expect to find evidence of altered regulation of T cell responses before ALS development. To address this hypothesis, we have used population-based data from a compulsory Norwegian tuberculosis screening program, including a quantitative measure of a post-vaccination immune response against Bacillus Calmette–Guérin (BCG). In people having undergone BCG vaccination, the tuberculosis skin test (TST) represents an objective and quantifiable measure of an adaptive immune response to a recall antigen. It is regarded as a classical delayed hypersensitivity reaction, involving antigen uptake and presentation by dendritic cells, activation and attraction of T cells, and induction of macrophages (Kobayashi et al., 2001). A minority of healthy people do not develop a positive TST after BCG vaccination but are nevertheless equally well protected against tuberculosis disease (and less to infection) (Simmons et al., 2018). The molecular and cellular mechanisms that govern TST responses after BCG vaccination involve immune cells also relevant in ALS, including interferon gamma producing memory T cells (Black G et al., 2001; Kowalewicz-Kulbat et al., 2018), Th17 cells, Treg (Gopal et al., 2012a), plasmacytoid dentritic cells (Bond et al., 2012) and monocytes (Simmons et al., 2021).

We designed a cohort study where participants had undergone a standardized immunization (BCG vaccine) followed by a TST, and aimed to disclose any association between young adulthood immune responses and long term ALS risk.

2. Materials and methods

2.1. Study population

In the beginning of the twentieth century Norway had one of Europe's highest rates of tuberculosis (Bjartveit and Waaler, 1965). Responding to this threat, and equipped with new possibilities for case finding, vaccination and treatment, the Norwegian government initiated a compulsory national tuberculosis screening program in 1948 (Liestøl et al., 2007). In repetitive rounds, citizens were tested with TST and chest x-rays. TST negative persons aged 15–40 years with no signs of tuberculous disease were highly encouraged to accept BCG vaccination. BCG was at the same time introduced in the national school vaccination program, resulting in high BCG coverage in the school-leaving cohorts. By the time of the screening initiation, tuberculosis rates had already dropped substantially, and became almost negligible during the second half of the century (Bjartveit and Waaler, 1965).

Gradually implemented, the mass screening program was nationwide from 1952 (Bjartveit, 1997) and lasted until 1975. Data from all counties except Oslo were entered in a central database. About 300,000–400,000 inhabitants (≈10% of the Norwegian total population) were tested annually, with mobile fluoroscopes on boats and buses used

to reach remote areas. Attendance was compulsory for all individuals aged 15 years and above, resulting in an overall attendance rate of about 85 % (Waaler, 1984). Non-attendance was mainly due to "acceptable excuses", such as in military service or in hospital, or already under control or treatment for tuberculosis (Waaler, 1984). Objective measurement of height and weight was included from 1963, following evidence showing that low body mass index (BMI) had a considerable predictive value for tuberculosis (Edwards et al., 1971). Participants who could document a positive TST without prior BCG vaccination were recorded as "converters" (infected) and were neither offered BCG vaccination nor retested. Also individuals previously BCG vaccinated underwent TST in this screening program, documenting the immunological response to the vaccine.

Computerized data from the last round (1963–1975) of this screening program are available, and contains information on 1,911,598 individuals. Citizens born in 1955 were the last to have complete coverage within the screening program.

From the screening program data, we first identified participants born from 1910 to 1955. Thus, they were young enough to be eligible for BCG vaccination either through the tuberculosis screening program from 1948 (where all under 40 were offered vaccine) or the school vaccination program if they showed no signs of infection. We included only those documented as BCG vaccinated. We excluded participants with missing or uncertain TST or BCG status information. Only calendar year for vaccination and TST were available. To ensure that the vaccination had preceded the TST, we limited participants to those with a previously documented BCG vaccination at least a year before examination with a TST. Over time immune responses induced by the vaccine typically wanes as well as risk of confounding from superinfection and comorbidity increases. Still, in populations with low tuberculosis rates, it has been shown that in the following 15 years after BCG vaccination given after infancy, the variation in TST reactivity most probably reflect individual variation in T-cell mediated immune responses to BCG rather than environmental exposure to mycobacteria (Geiter et al., 1996; Wang et al., 2002; Weir et al., 2008). Beyond that period, the BCG-dependent TST reactivity is more uncertain (Weir et al., 2008). Therefore, we restricted participants to those with less than 15 years between BCG and TST. Ultimately, we excluded participants with suspected lung tuberculosis. From 1962, patients diagnosed with tuberculosis were reported to "The Central Tuberculosis Register" (Bjartveit, 1997). The diagnosis was based on bacteriological confirmation or clinical assessment usually with x-ray and a decision to start treatment. We linked information from The Central Tuberculosis Register to the screening cohort, and excluded those with confirmed tuberculosis. We also excluded a small number of participants with suspected lung tuberculosis based on lung or pleural infiltrates or calcifications on chest x-ray documented in sceening cohort data. These selections constituted our main study cohort (Fig. 1).

We further identified a sub cohort of participants born from 1935 to 1955 who were all vaccinated at age 12–17 years. In this sub cohort, risk of super infection was even lower than in the main cohort and their history of exposure to mycobacterial antigens were more homogenous.

2.2. Exposure

From the tuberculosis screening data, we collected information on year and month of birth and screening, sex, county of residence, vaccination status and year of vaccination, TST status and induration size (in mm), x-ray findings and results from objective measurements of height and weight.

For TST, the adrenaline-pirquet test was used (Jentoft et al., 1999), which was the standard tuberculin skin test in Norway until 2004. Two drops of concentrated tuberculin (Old tuberculin) mixed with 1 % adrenalin was placed on the volar aspect of the underarm. Two 5-mm-long scratches through each drop was then produced in the superficial layer of the skin by means of a pen nib. After 2–3 days, the largest infiltrate was measured in millimeters according to strict
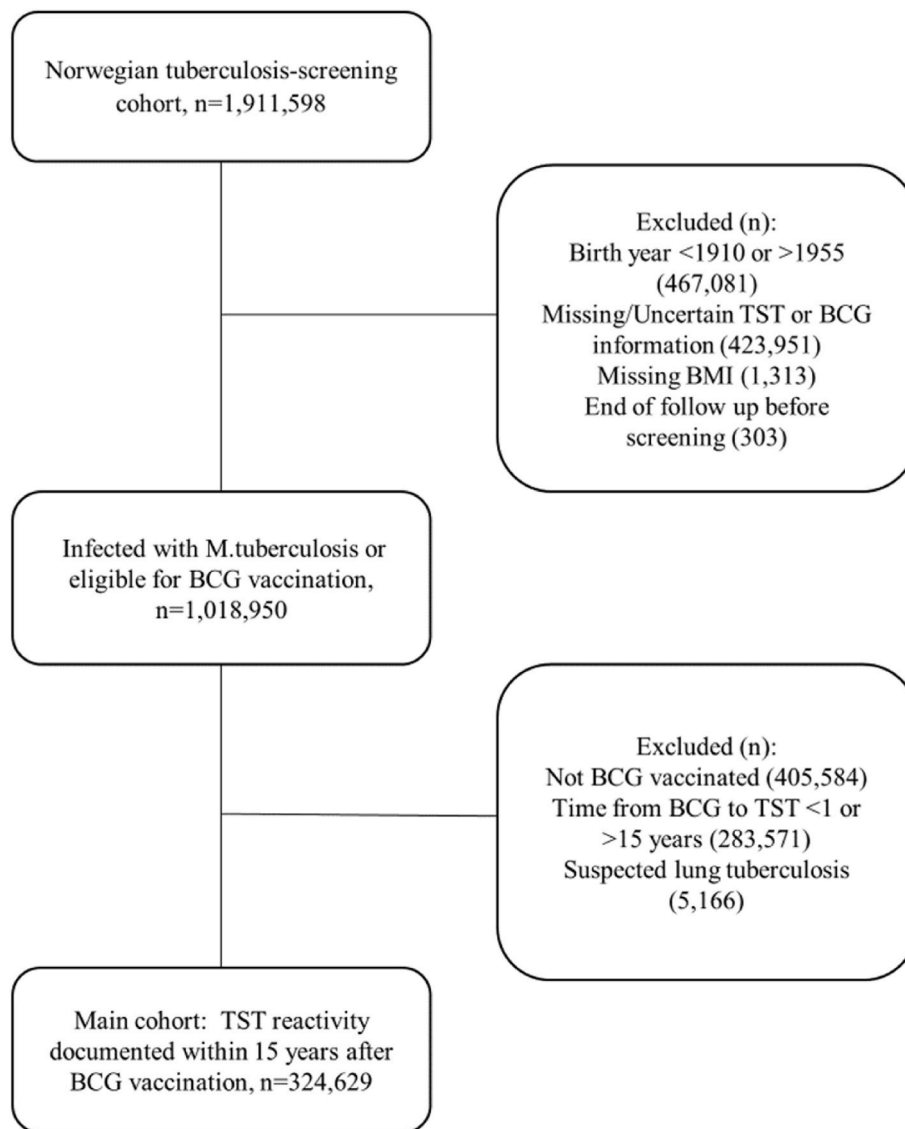
Fig. 1. *Selection of participants* Abbreviations: BCG= Bacille Calmette Guerin, TST = Tuberculin Skin Test, BMI= Body Mass Index.

national guidelines (Waaler et al., 1975). Thus, to reduce the potential of systematic error, nurses and doctors underwent centralized training in the testing and reading techniques, and bio-assays of current tuberculin batches were carefully controlled. The adrenaline-pirquet test has demonstrated reproducibility similar to the internationally recognized Mantoux test (Jentoft et al., 1999).

BCG was produced at the Bergen State BCG Laboratory (Bergen, Norway) using the Swedish Gothenburg strain until 1973 (Hesselberg, 1972) and thereafter by Statens Serum Institute (Copenhagen, Denmark). Liquid BCG was gradually replaced by freeze-dried BCG between 1959 and 1973 (Tverdal and Funnemark, 1988).

2.3. Case ascertainment

ALS cases were identified through the Norwegian Cause of Death Registry and the Norwegian Patient Registry. The Norwegian Cause of Death Registry collects and processes all death certificates in Norway. Available digitalized data from 1951 onwards contains information about the direct, contributing and underlying causes of death, coded according to different revisions of the International Classification of Diseases (ICD). We collected data on all ALS deaths from the start of the last round of the tuberculosis screening program (1963) to June 2020.

ALS was defined as ICD codes corresponding to motor neuron disease mentioned anywhere on the death certificate. We used the following codes; ICD 7 (1963–1968): 360.0 and 360.1; ICD 8 (1969–1985): 348.0, 348.1, and 348.2; ICD 9 (1986–1995): 335.2; ICD10 (1996 onwards): G12.2. In a previous study, we have shown that the Norwegian Cause of Death Registry has high sensitivity for ascertaining ALS (Nakken et al., 2016).

The Norwegian Patient Registry is an administrative health register containing information on all in- and outpatient admission from Norwegian hospitals and private practice specialists with public reimbursement. Registered ICD-10 codes and dates from each patient visit are automatically transferred to the Patient Registry. Individual data are available from March 1, 2007. We defined ALS cases as those who had two or more G12.2 (ICD-10) entries in the registry, with date of incidence set to first entry. This method is validated and found reliable in a previous study (Nakken et al., 2018). We collected Patient Registry data from January 2008 until January 2018. If the ALS cases were present in both the death registry and the patient registry, the death registry was chosen. Out of the total 496 cases retrieved, 231 was found in both registries, only 11 exclusively in the Patient Registry.

We retrieved vital status and emigration from the National Population Registry. Since 1964, every Norwegian citizen has a unique

personal identification number, allowing linkage between registries.

2.4. Statistics

We dichotomized and compared secondary immune responses following vaccination adopting the definition used for positive (≥4 mm) or negative TST (<4 mm) during the screening program (Waaler et al., 1975). We used positive TST as reference. In order to improve confidence that measured TST indeed reflected secondary immune responses to BCG vaccination, we performed additional analyses where the sample was limited to individuals with less time between BCG and TST.

In our sub cohort of young vaccinees born after 1935, we modelled immune responses following BCG-vaccination as for the main cohort.

Each participant contributed follow-up time from the date of tuberculosis screening (1963–75) to the date of ALS (death or diagnosis), death from other causes, emigration or the end of study follow up (May 31st' 2020), whichever came first. Hazard ratios (HR) and their 95 % confidence intervals (CI) were calculated using Cox proportional hazard models with attained age (age at screening + time since screening) as time variable. Sex, birth year (continuous) and screening year 1963–75 (categorical, 3 year bins), BMI (continuous) and county (categorical) was included as covariates in our fully adjusted model. To assess potential time-varying effects during follow-up, we fitted flexible parametric models using the stpm2 package in Stata (Royston and Parmar, 2002). Here, we allowed the effect of having a negative TST to vary over analysis time, using a spline. We then calculated and plotted predicted HRs, using time since screening as analysis time. Between sexes heterogeneity was tested using a likelihood ratio test between the multivariable adjusted model and a model including an interaction term of exposure categories and sex. Also, to minimize the possibility of including participants in an early clinical phase, the first 10 years of follow-up were excluded in sensitivity analyses.

3. Results

Our main cohort comprised 324,629 participants (52 % women), collectively contributing 15,436,614 person years of observation time. Overall, median age at screening was 22 (interquartile range (IQR) 10) years and median follow up time was 51 (IQR 6) years. Median time between BCG vaccination to TST (screening) was 8 (IQR 7) years. During a median follow up time of 40 (IQR 11) years, we identified 496 ALS cases (50 % women). Baseline characteristics of our main cohort are given in Table 1.

Having a negative TST was associated with reduced ALS risk (hazard ratio (HR) 0.75 (95% CI 0.58–0.97)) (Table 2). This association remained unchanged during follow up and was present more than 4 decades after TST measurement (Fig. 2). The strength of the association between a negative TST and reduced ALS risk increased with less time between vaccination and TST. Sensitivity analyses excluding the first ten years of follow-up did not change our results (Table 2). There were no

Table 1
Baseline characteristics for main cohort, according to ALS-status and sex. Abbreviations: TST = Tuberculin skin test, BCG=Bacillus Calmette Guerin, BMI= Body mass index, IQR = Interquartile range, SD= Standard deviation.

	Men		Women	
	ALS	Non-ALS	ALS	Non-ALS
Participants, N	248	155,516	248	168,617
Birth year, median (IQR)	1942 (13)	1947 (10)	1946 (11)	1943 (17)
Age at TST, years, median (IQR)	26 (11)	22 (10)	26 (14)	22 (10)
Age at BCG, years, median (IQR)	17 (7)	13 (6)	15 (9)	13 (6)
BMI, kg/m², mean (SD)	23.2 (2.8)	22.7 (2.9)	23.0 (3.3)	22.5 (3.4)

clear differences between sexes (p = 0.11).

In a sub population comprising 228,900 individuals (52 % women) born 1935–1955 (median 1949 (IQR 7)) who were all BCG-vaccinated in adolescence between 1947 and 1972, when *M tuberculosis* infection rates were low, 275 (54 % women) ALS cases were identified with a median follow up of 43 (IQR 9) years. Median age at screening was 19 (IQR 7) years and 83 % had positive TST. Median time from BCG to TST was 7 (IQR 6) years. Results were concordant with results from the main study cohort, with effect estimates somewhat larger in the sub cohort. Thus, HR for ALS was 0.67 (95% CI 0.46–0.96) in the group with negative TST compared to the group with positive TST (Supplementary Table 1). As for the main cohort, there was no effect modification by sex (p = 0.78). In sensitivity analysis excluding the first ten years of follow-up only 1259 participants (no ALS cases) were left out from the original sub cohort. Consequentially, results remained unchanged.

4. Discussion

In the current study, we used a population based approach to investigate whether regulation of a T cell mediated immune response in young adulthood relates to ALS risk. Using data from a national tuberculosis screening program, we here show an association between weak tuberculin reactivity following BCG vaccination and long term decreased risk of ALS. The observed association persisted for decades after screening, ruling out reverse causality.

The TST provides an *in vivo* model for studying regulation of a T cell mediated immune response at a population level. It involves T cell subsets that are also recruited to ALS lesions and that display clonal expansion and differences in activation status in blood and CSF of ALS patients (Jin et al., 2020; Yazdani et al., 2022). Thus, it has been shown that subsequent TST reactivity after BCG vaccinatioin is related to IFN gamma-producing Th1 memory T cells and regulatory T cells (Kowalewicz-Kulbat et al., 2018; Serrano et al., 2015), and that Th17 cells facilitate BCG responses (Gopal et al., 2012b). Conversely, increased frequencies of activated Th1 and Th17 cells concomitant with diminished immune regulation by Tregs found in ALS patients suggest an involvement of these immune cells also in ALS (Rolfes et al., 2021; Yazdani et al., 2022).

Other explanations must be considered, including mycobacterial infection as a detrimental factor on the motor system and its supporting cells. Although not explored for mycobacterial agents, other infections might contribute to protein aggregation and mislocalization as well as glutamate excitotoxicity—known pathological processes of ALS (Lotz et al., 2021). In our study, a positive TST following BCG vaccination could represent superinfection with *M tuberculosis* or atypical mycobacteria. However, the association between ALS risk and post-BCG tuberculin reactivity was most marked with restricted time between vaccination and TST and in our sub cohort born in an era when tuberculosis infection was rare. If superinfection wth tuberculosis or other mycobacteria explained the observed association, one would expect larger effect sizes with increasing time from vaccination to TST and in older birth cohorts, whereas we observed the opposite. Ultimately, while both general infections and tuberculosis have been associated with increased risk of Alzheimer disease and Parkinson disease, this has not been the case for ALS (Shen et al., 2016; Sun et al., 2022). Taken together, mycobacterial infection per se is therefore a less likely explanation of our results.

Our study has limitations. As for all observational studies, we cannot exclude bias from unmeasured confounding factors. We lack information on smoking, which can increase tuberculosis infection risk (Maurya et al., 2002) and affect immune responses to vaccination (Zimmermann and Curtis, 2019). Any link between smoking and ALS risk is however more uncertain (Opie-Martin et al., 2020; Zhan and Fang, 2019). The association between secondary immunity to vaccination and ALS risk was equal between sexes, however smoking was far less prevalent among women in relevant birth cohorts (Ronneberg et al., 1994).

Table 2
Risk (HR) of ALS by categories of immune responses following BCG vaccination in Norwegian citizens born 1910–1955.

Tuberculin skin test reactivity	Participants	Cases	ALS mortality rate pr 100,000 person years (95% CI)	Multivariable adjusted[a] HR (95% CI)	Multivariable adjusted[a] HR (95% CI) excluding ALS within 10 years
BCG vaccinated within 15 years prior to TST					
Positive	265,908	426	3.4 (3.1–3.7)	Reference	
Negative	58,721	70	2.5 (2.0–3.2)	0.74 (0.57–0.95)	0.75 (0.58–0.97)
BCG vaccinated within 10 years prior to TST					
Positive	162,007	201	2.5 (2.2–2.9)	Reference	
Negative	40,725	35	1.8 (1.3–2.4)	0.67 (0.46–0.96)	0.67 (0.47–0.97)
BCG vaccinated within 5 years prior to TST					
Positive	70,942	76	2.1 (1.7–2.7)	Reference	
Negative	18,117	12	1.3 (0.8–2.3)	0.57 (0.31–1.06)	0.57 (0.31–1.06)

[a] Adjusted for birth year, screening year, BMI, county of residence and sex, using attained age as timeline. Abbreviations: BCG= Bacille Calmette-Guérin, TST = Tuberculin skin test, HR= Hazard Ratio, CI= Confidence Interval.
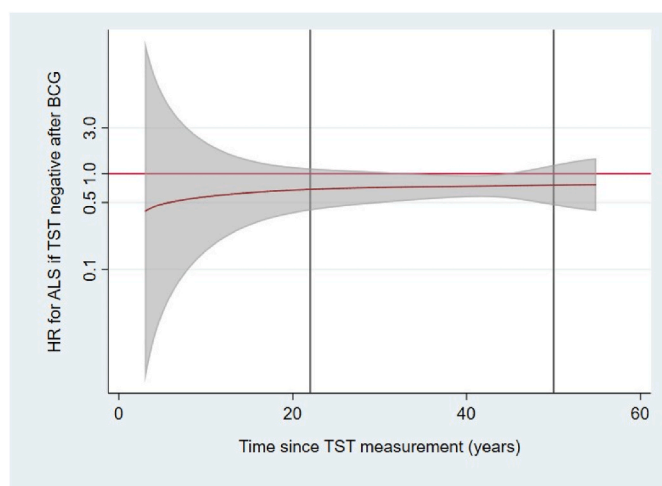
Fig. 2. *Risk (hazard ratio (HR)) of amyotrophic lateral sclerosis (ALS) according to negative tuberculin response following BCG.* ALS risk plotted against time since tuberculin skin test (TST), adjusted for age, birth year, sex and body mass index. 95% confidence intervals in grey. Vertical lines indicate the 5th and 95th percentile of the ALS case distribution.

Therefore, confounding by smoking is less likely. Our cohort does not include genetic or phenotypic information on ALS cases, making stratified analyses impossible. The study comprises a rather homogenous cohort of ethnic Norwegian citizens and should be generalizable to other Caucasian populations. Ultimately, the vast majority of cases were ascertained through death records (mortality). When using mortality as proxy for incidence, the association observed could in part be due to differential survival across exposure categories. Still, ALS mortality data have good accuracy for incidence rates (Marin et al., 2011), also in Norway (Nakken et al., 2016). Mean survival time for ALS is short, and most participants had several decades of follow-up. We therefore do not think mortality data has confounded our results.

We conclude that on population level, a weak secondary adaptive immune response, as measured by TST following BCG vaccination, is associated with low ALS risk several decades later. These results support a primary role for immune regulation in ALS development.

Funding

Ethics

The study was approved by the regional ethics committee (REC South East, ref. no 2016/1731), with informed consent waived in the current study as the risk of re-identification was considered minimal as well as any possible privacy invasion. Using pseudonymized data, the present analyses did not require additional permissions. The present report follows STROBE guidelines for the presentation of original epidemiological research.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The authors do not have permission to share data.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbih.2023.100704.

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