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DNA methylation patterns associated with prior tuberculosis infection in people with HIV

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Mechanisms by which prior tuberculosis (TB) increases long-term risk for cancer, cardiovascular, and neurological disorders remain unclear, particularly in people with HIV (PWH). This study investigated DNA methylation (DNAm) patterns and associated pathways in PWH with and without prior TB infection. DNAm was analyzed in blood samples from 30 PWH (10 with prior latent TB infection [LTBI], 10 with previous successfully treated active TB, and 10 with no TB) using the Illumina MethylationEPIC BeadChip covering over 850,000 CpG sites. Epigenetic age was estimated, and age acceleration was calculated. Differentially methylated CpGs (dmCpGs) and regions (DMRs) were identified, and functional enrichment analyses for Gene Ontology, KEGG pathways, PANTHER database, and gene set enrichment analysis (DisGeNET, dbGaP) were performed. Statistical significance was set at a false discovery rate (FDR) of < 0.05. PWH exhibited significant epigenetic age acceleration, with a mean of 19.32 ± 10.82 years greater than chronological age. This accelerated aging was more pronounced in individuals with any prior TB infection (21.60 ± 12.03 years) compared to those without TB (17.42 ± 9.38 years). In the prior active TB vs. no TB comparison, 7461 dmCpGs were identified, corresponding to 150 DMRs ($p < 0.05$), with top associated genes including GRAMD1C (hypomethylation), DPP6 (hypermethylation), and HDAC4 (hypomethylation). In the LTBI vs. no TB comparison, 8598 dmCpGs were observed, corresponding to 39 DMRs ($p < 0.05$), associated with genes such as PLEKHG5 (hypermethylation), STK32C (hypermethylation), and SPATC1L. When comparing any prior TB (active or latent) to no TB, 71,774 dmCpGs and 14 DMRs were identified, including genes like PLEKHG5, KCNN3, and BRSK2. Pathway analyses of prior TB (active or latent) vs. no TB revealed enrichment in neurogenesis, neuron differentiation, axon guidance, and neuroactive ligand signaling. Additional enriched pathways included those related to platelet activation, vascular muscle contraction, and chemokine signaling. Cancer-related pathways such as proteoglycans in cancer, small cell lung cancer, prostate cancer, breast cancer, hepatocellular carcinoma, and thyroid cancer were also enriched. PANTHER analysis showed consistent enrichment in the Wnt signaling pathway and inflammation-mediated pathways across compared groups. DisGeNET analysis linked prior TB DNAm patterns to lymphoid leukemia, while dbGaP analysis identified associations with phenotypes like asthma, body mass index, tunica media, and lymphocyte count. Prior TB infection in PWH is associated with distinct DNAm changes in pathways related to neural function, cardiovascular health, and cancer risk, and is linked to more pronounced epigenetic age acceleration, suggesting epigenetic mechanisms for TB-related long-term complications.

Keywords DNA methylation, TB, HIV, Cancer, Cardiovascular disease, Dementia

Abbreviations

ART	Antiretroviral therapy
CVD	Cardiovascular disease
DMR	Differentially methylated region
DNAm	DNA methylation
dmCpGs	Differentially methylated CpG sites
FDR	False discovery rate
HIV	Human immunodeficiency virus

KEGG	Kyoto encyclopedia of genes and genomes
LTBI	Latent tuberculosis infection
MsigDB	Molecular signatures database
NK	Natural killer (cells)
PANTHER	Protein analysis through evolutionary relationships
PWH	People with HIV
QFT	QuantiFERON
RMSD	Root mean squared deviation
TB	Tuberculosis

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Despite the improvement in diagnostic modalities and discovery of effective chemotherapeutic agents, tuberculosis (TB) is the leading cause of death from a single infectious agent, contributing 1.25 million deaths in 2023[1]. Although the global TB treatment success for drug sensitive TB is high, long-term complications of TB contribute to significant disability and mortality even after TB treatment success[2,3]. After completion of TB treatment, previously treated patients are 3 times more likely to die than individuals without TB, and this is independent of age, sex, country income and type of TB[4] or drug resistance profile[5]. For this reason, there are growing calls for programmatic follow up of TB patients after TB cure or treatment completion[6,7]. While these calls draw attention to the several post TB pulmonary complications resulting from structural changes in the lung (such as chronic obstructive airway disease, bronchiectasis, and fibrosis) [7], cardiovascular disease (CVD) and cancer account for almost 40% of deaths after TB treatment completion, while respiratory causes contribute only 14%[4]. Evidence from cohort studies shows that pulmonary TB (PTB) increases the risk for ischemic stroke[8], acute coronary syndrome[9], myocardial infarction[10], and chronic kidney disease[11]. Additionally, PTB has been reported to increase the incidence of neurologic complications even in the absence of central nervous system tuberculosis. Dementia[12], parkinsonism[13] and depression[14] are reported in population-wide cohort studies. Finally, TB is associated with an increased risk for cancer at ten sites[15]. Latent TB infection (LTBI) has been associated with similar risks for cancer, CVD and mental health problems[16–18]. There is an urgent need to understand the mechanisms underlying long term complications of active and latent TB.

The mechanisms underlying the long-term complications of TB are not well elucidated. Chronic inflammation and traditional CVD risk factors such as diabetes mellitus, smoking, and age have been implicated in increasing CVD risk[19,20]. Active TB and LTBI could contribute to cancer development by inducing chronic inflammation, genomic instability, and modulation of host cell signaling pathways[21]. Further, DNA methylation is thought to occur in genes and pathways involved in immune-regulation even after TB treatment. For example, this has been observed in genes involved in cytokine production (Interleukin (IL)-6), toll-like receptor signaling (TLR2), and other immune-related pathways (PI3K-AKT, MAPK, mTOR)[22]. Moreover, people with prior active TB have been shown to have an increase in the DNA methylation age by 13 years above the chronological age while cellular aging is increased by 14 years among people with LTBI[23]. However, these mechanisms have been demonstrated in predominantly HIV negative cohorts despite evidence that people with HIV (PWH) already experience ongoing accelerated aging driven by persistent inflammation, immune senescence, mitochondrial dysfunction, epigenetic alterations, and long term toxicities attributed to anti-retroviral therapy (ART) [24]. There is a paucity of studies that have evaluated DNA methylation patterns associated with prior TB in PWH.

Our study aim was to determine whether prior TB infection is associated with DNA methylation (DNAm) patterns and pathways that could confer future risk for long term complications among PWH. By elucidating these mechanisms, the findings could inform targeted interventions to mitigate long-term complications, offering new avenues for improving the care of individuals with TB-HIV coinfection.

Methods

Study design and population

In this cross-sectional study, 30 adult PWH on ART were randomly selected from three HIV clinics in Uganda. Detailed study methods are described elsewhere[25]. Participants were stratified into three groups: 10 with prior LTBI, 10 with previously treated active TB, and 10 with no history of TB infection. Previous treatment for active TB (diagnosed either by sputum GeneXpert, microscopy or urine lipoarabinomannan) was ascertained from the HIV care records and the unit TB register at the respective clinics. LTBI was defined as a positive Quantiferon (QFT)-plus assay, according to manufacturer's instructions, in an individual without previous treatment for active TB[26]. PWH with LTBI had all completed a course of TB preventive therapy according to national guidelines prior to enrollment[27]. Prior TB was defined as either LTBI or previous treated active TB. PWH with no TB had never been treated for active TB and had a negative QFT-plus assay.

Sample collection and processing

Whole blood samples (5 ml) were collected from each participant using EDTA vacutainers. Genomic DNA was extracted from the blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. DNA quantity was measured using Qubit 4 fluorometer (ThermoFisher Scientific, USA), following the manufacturer's instructions. For the methylation assay, 500 ng of DNA was used. DNA methylation (DNAm) levels were assayed using the Illumina Infinium MethylationEPIC v2.0 BeadChip array and the iScan system (Illumina, Inc., San Diego, CA, USA), in accordance with the manufacturer's instructions, generating IDAT files for downstream bioinformatics analysis to assess methylation levels.

Data processing and analysis

The raw data files obtained from the Illumina MethylationEPIC BeadChip were preprocessed and analyzed using the minfi package in the R statistical software environment. The minfi package is a comprehensive toolkit specifically designed for the analysis of Illumina Infinium methylation arrays[28]. The preprocessing steps involved an initial quality assessment of the raw data using various metrics, including visualization of beta value distributions and evaluation of control probes to ensure optimal bisulfite conversion efficiency. Subsequently, background correction and quantile normalization were performed using the preprocess Quantile function to minimize technical variation and ensure consistent methylation value distributions across samples. To ensure data quality, probes with a detection *p*-value greater than 0.01 were removed, and probes located on sex chromosomes (X and Y) were excluded to avoid confounding due to sex differences. Additionally, probes containing single nucleotide polymorphisms (SNPs) were removed using the dropLociWithSnps function to prevent methylation differences caused by genetic variation.

Blood cell composition was estimated using the estimateCellCounts2 function, referencing the FlowSorted.CordBloodCombined.450 k dataset and utilizing the IDOLOptimizedCpGsCordBlood marker set. The cell types assessed included CD4+ T cells, CD8+ T cells, B cells, monocytes, natural killer (NK) cells, granulocytes, and nucleated red blood cells. The consistent proportion of blood cell types across samples, as detailed in Supplementary Table 1, indicated the absence of biological bias in the experimental setup. This consistency is important because the selected Horvath's methylation clock, used for age estimation, incorporates various cell types, including blood cells.

Methylation beta values were also used to estimate epigenetic age for each individual using PC-PhenoAge, a second generation methylation clock able to predict mortality[29]. Age acceleration represented the extent to which the biological age of an individual exceeds their chronological age at the time of measurement. Age estimates were determined using dnaMethyAge package version 0.2.0 and the measure for age acceleration was defined as residuals yielded from regressing epigenetic age on chronological age from which root mean squared deviation (RMSD) and mean absolute deviation (MAD) were computable.

Identification of differentially methylated CpGs and regions

Differentially methylated CpGs (dmCpGs) and regions (DMRs) were identified using the dmpFinder function in minfi, which applies a linear model to compare methylation levels while adjusting for age and sex, methylation batch effect and cell-type covariates. This function performed pairwise comparisons between the three groups (LTBI, active TB, and no TB) to identify CpG sites that exhibit significant differences in methylation levels. Significant regions were defined by β-value differences >0.2 and permutation testing ($B=0$). The dmpFinder function utilizes statistical tests to assess the significance of methylation differences, adjusting for multiple comparisons to control the false discovery rate (FDR). An FDR <0.05 was considered statistically significant. The Comparisons included: 1. Previous Active TB vs. no TB; 2. Prior LTBI TB vs. no TB; 3. Prior TB infection (both LTBI and previous active TB) vs. no TB; and 4. Previous active TB vs. LTBI.

Pathway analysis

Pathway and gene ontology (GO) enrichment analyses were conducted using the missMethyl package to identify biological pathways associated with significant DMRs. These included GO enrichment analysis[30] using the gometh function and pathway analysis through the gsameth function, leveraging gene sets from the Kyoto Encyclopedia of Genes and Genomes (KEGG)[31], Molecular Signatures Database (MSigDB)[32] and PANTHER version 16 database[33]. We further performed gene-set enrichment analysis of our 120-gene signature using the Enrichr web server (http://amp.pharm.mssm.edu/Enrichr; accessed June 1, 2025)[34]. Up-regulated genes (FDR <0.05, $|\log_2 FC|>1$) in prior-TB versus TB-naïve samples were submitted and tested against both the DisGeNET library of curated gene–disease associations (v7.0) and the dbGaP library of GWAS-derived phenotype associations. For each term, Enrichr computes a Fisher's exact test *p*-value, Benjamini–Hochberg–adjusted *p*-value (FDR), odds ratio, and combined score ($-\log_{10} p \times$ z-score of deviation from expected rank). We considered terms with FDR <0.05 as significant and report, for all hits, the gene overlap, odds ratio, adjusted *p*-value, and combined score.

All statistical analyses were carried out using R version 4.3.1.

Results

Characteristics of study participants of PWH with and without prior TB

Of 30 participants, 17 (57%) were female and the median (interquartile range, IQR) age was 46.5 (40.0–50.0) years. All participants were on ART and had achieved viral load suppression (viral load <1000 copies/ml). Table 1 shows characteristics of the participants.

Characteristic	Total (N = 30), (%)	Prior active TB (n = 10), (%)	Prior latent TB infection (n = 10), (%)	No prior TB (n = 10), (%)	*p*-value
Age, median (IQR), (years)	46.5 (40.0–50.0)	47.5 (47.0–51.0)	42.0 (36.0–51.0)	45.5 (40.0–47.0)	0.36
Female sex	17 (57%)	5 (50%)	7 (70%)	5 (50%)	0.58
Duration on ART, median (IQR) (months)	144.0 (117.0–168.0)	144.0 (117.0–197.0)	144.0 (120.0–153.0)	144.0 (117.0–168.0)	0.99
Any history of alcohol use	17 (57%)	5 (50%)	5 (50%)	7 (70%)	0.58
Any history of cigarette smoking	3 (10%)	1 (10%)	1 (10%)	1 (10%)	1.00
Use of biomass fuel for cooking	18 (60%)	7 (70%)	5 (50%)	6 (60%)	0.66
Baseline CD4 Count at HIV diagnosis, median (IQR) (cells/mm^3)	208.0 (47.0–405.0)	217.5 (69.0–420.0)	184.0 (0.0–350.0)	136.0 (47.0–405.0)	0.72
Time since Treatment completion, mean (SD) years	N/A	12.0 (6.1)			
Current CD4 Count at study enrolment, median (IQR) (cells/mm^3)	911.0 (691.0–1374.0)	847.5 (548.0–1357.0)	1141.5 (875.0–1451.0)	875.0 (589.0–1180.0)	0.50
Haemoglobin level, median (IQR), (g/dl)	15.0 (14.3–16.0)	15.2 (14.7–16.3)	14.4 (13.4–15.0)	15.6 (14.3–16.1)	0.15
Aspartate aminotransferase, median (IQR) (IU/L)	26.9 (23.5–33.5)	26.8 (23.4–33.5)	27.4 (24.7–31.4)	27.9 (22.3–35.3)	1.00
Gamma-glutamyl transferase, median (IQR), (IU/L)	46.5 (42.6–58.5)	44.8 (30.1–57.6)	45.6 (42.6–56.9)	51.9 (44.6–61.2)	0.37
Alanine aminotransferase, median (IQR), (IU/L)	22.2 (16.7–31.5)	19.7 (16.0–26.0)	23.6 (16.4–32.7)	23.4 (20.1–32.8)	0.55
Alkaline phosphatase, median (IQR) (IU/L)	114.0 (85.0–152.0)	109.5 (89.0–141.0)	120.0 (81.0–163.0)	119.5 (85.0–152.0)	0.94
Albumin, median (IQR), (g/l)	4.2 (3.4–5.8)	4.5 (3.4–6.3)	3.6 (2.2–4.9)	4.4 (3.6–5.6)	0.50
Total protein, median (IQR)(g/l)	7.7 (7.1–8.3)	7.6 (7.4–8.0)	7.5 (6.9–7.8)	8.3 (7.1–8.6)	0.13
Urea, median (IQR), (mmol/l)	3.7 (2.8–4.7)	3.7 (2.9–4.2)	4.4 (3.1–4.8)	2.9 (2.5–4.6)	0.39
Creatinine , median (IQR), (mmol/l)	113.0 (96.1–126.5)	113.0 (98.1–126.5)	104.7 (92.6–129.2)	118.5 (96.1–124.4)	0.88
Serum Uric acid, median (IQR), (µmol/l)	265.5 (195.0–370.6)	341.2 (261.2–454.8)	222.1 (195.0–287.2)	300.2 (36.1–370.6)	0.14
Serum lactate dehydrogenase, median (IQR), (U/L)	595.5 (490.0–780.0)	636.5 (450.0–882.0)	605.0 (555.0–689.0)	513.0 (430.0–751.0)	0.43

Table 1. Characteristics of study participants.

Chronological and epigenetic age of in PWH with and without prior TB

In the combined 30 PWH (mean chronological age 43.6 ± 9.3 years), PC-PhenoAge estimates averaged 62.95 ± 10.65 years, yielding a mean epigenetic age acceleration of 19.32 ± 10.82 years. Overall, epigenetic age exceeded chronological age by a highly significant margin (paired t(29) = 9.77, $p < 0.001$, 95% CI 15.28–23.36).

Among the 10 TB–naïve individuals the mean chronological age was 43.8 ± 8.5 years while among the 20 TB survivors it was 44.3 ± 8.8 years. PC-PhenoAge estimates averaged 61.2 ± 10.7 years in the TB–naïve group and 65.9 ± 9.2 years in the prior-TB group. Within the TB–naïve cohort, epigenetic age significantly exceeded chronological age by a mean of 17.42 ± 9.38 years (paired t(9) = 5.87, $p < 0.001$; 95% CI 10.71–24.14). Similarly, among participants with a prior TB infection, PC-PhenoAge exceeded true age by 21.60 ± 12.03 years (paired t(19) = 8.03, $p < 0.001$; 95% CI 15.97–27.23). The mean epigenetic-age acceleration was 4.18 years greater in TB survivors compared with TB–naïve individuals (95% CI: −4.13 to 12.49). However, this difference did not reach statistical significance (Welch's t(22.6) = 1.04, $p = 0.307$), and the effect size (Cohen's d ≈ 0.37) indicates only a small-to-moderate group separation, suggesting that a larger sample may be required to confirm this trend.

The comparison of epigenetic and chronological age between prior TB infection (both LTBI and active) and those without TB is shown in Fig. 1. The results demonstrate an overall trend toward accelerated aging among PWH that was more pronounced in people with prior TB infection (both LTBI and active TB).

DNA methylation patterns in PWH with and without prior TB

A total of 7461 differentially methylated CpGs (dmCpGs) were identified in the comparison between prior active TB vs. no TB groups. These dmCpGs corresponded to 150 differentially methylated regions (DMRs) with $p < 0.05$, associated with the following top genes: GRAMD1C (hypomethylation), DPP6 (hypermethylation), ANKRD53 (hypomethylation), HDAC4 (hypomethylation), ADAMTS16 (hypomethylation), FAM208B (hypomethylation), BFSP2(hypomethylation), ZNF665 (hypermethylation), DOCK1 (hypermethylation), and PRDM16 (hypermethylation) (Supplementary Table 2 and Fig. 2: A1&A2).

In the comparison between LTBI vs. no TB, 8598 dmCpGs were observed. These were associated with 39 DMRs at $p < 0.05$, including genes such as PLEKHG5 (hypermethylation), STK32C (hypermethylation), MCF2L (hypermethylation), PTPN6 (hypermethylation), SAMD11 (hypermethylation), AP001468.58 (hypomethylation), EPCAM (hypermethylation), NEURL4 (hypomethylation), SLC25A31 (hypomethylation), DNMT3A (hypomethylation), TTC7B (hypomethylation), FAM208B (hypermethylation), BRSK2 (hypermethylation), as well as the SPATC1L gene region with both hypo and hypermethylated probes (Supplementary Table 2 and Fig. 2: B1&2).

Overall, 71,774 dmCpGs were identified when comparing individuals with any history of TB infection (both active and latent TB) to the control group. These dmCpGs corresponded to 14 DMRs associated with genes such as PLEKHG5 (hypomethylation), KCNN3 (hypermethylation), SLC16A9 (hypomethylation), BRSK2 (hypermethylation), MCF2L (hypermethylation), NUP98 (hypomethylation) and hypermethylated SH3GL2. (Supplementary Table 2 and Fig. 2: C1&2). Meanwhile even though only two DMRs (PLEKHG5 and KCNN3) in this comparison were active TB vs No TB and prior latent TB vs No TB comparisons.

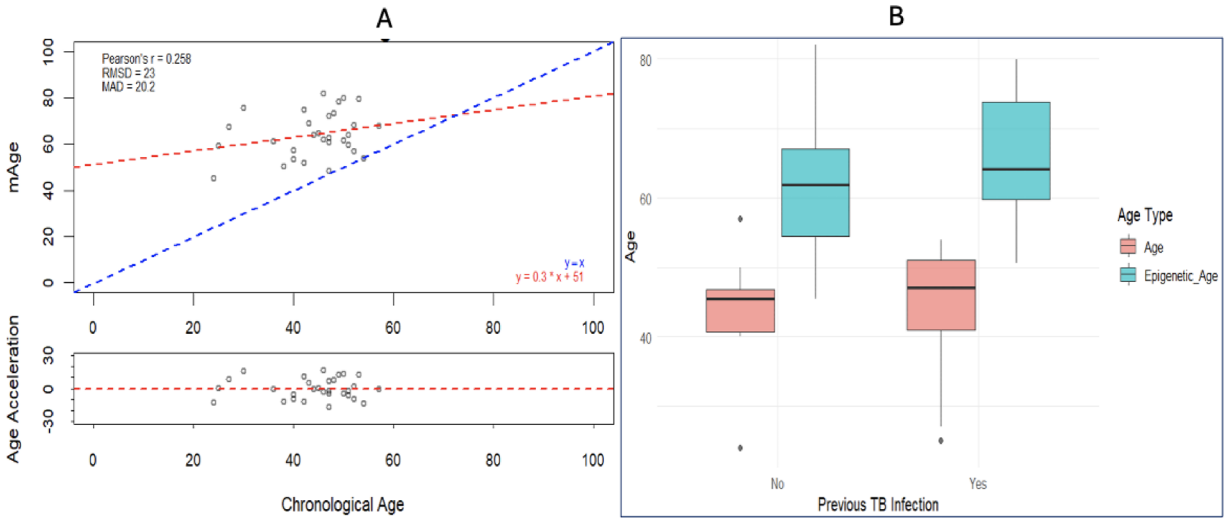

Fig. 1. Epigenetic Age (DNAm) Compared to Chronological Age in Individuals with and without Prior Tuberculosis Infection. (**A**) Scatter plot of DNAm age (y-axis) versus chronological age (x-axis) estimated using using PCPhenoAge methylation clock. The blue dashed line represents perfect agreement between DNAm age and chronological age, while the red dashed line represents the regression line derived from the actual data. Most samples are above the blue line, indicating an overestimation of DNAm age. The bottom panel shows age acceleration values (DNAm age minus chronological age residuals), with positive values indicating accelerated aging (DNAm age > chronological age) and negative values indicating decelerated aging (DNAm age < chronological age). The results demonstrate an overall trend toward accelerated aging. (**B**) Box plot comparing DNAm age (Epigenetic_Age) and chronological age (Age) between individuals with previous TB infection and those without TB infection. Both groups show epigenetic ages that exceed their true age, but this over-estimation is more pronounced in those with prior TB infection.

Enriched pathways associated with DNAm patterns in PWH with and without prior TB

Gene ontology, KEEG and human C2 GSEA

In the prior TB (active or latent) vs. no TB comparison, DNAm changes were enriched in pathways related to neurogenesis, neuron differentiation, axon guidance, neuroactive ligand signaling, morphine addiction, and nervous system development. Additional pathways related to platelet activation, vascular muscle contraction, chemokine signaling, and cytokine-cytokine receptor interaction. Further, pathways relating to cancer, proteoglycans in cancer, small cell lung cancer, prostate cancer, lung cancer, breast cancer, hepatocellular carcinoma, and thyroid cancer were enriched (Fig. 3A).

The other pairwise comparisons (prior active TB vs. no TB and prior LTBI vs. no TB comparisons) are shown in Fig. 3B and C but they did not meet statistical significance**.**

PANTHER

Analysis using the PANTHER database consistently showed enrichment in the Wnt signaling pathway, with 38 associated genes in the active TB vs no TB comparison and 41 genes in the latent TB vs. no TB comparison. Pathways involved in inflammation mediated by chemokine and cytokine signaling, integrin signaling, angiogenesis, Cadherin signaling pathway, CCKR signaling pathway and Nicotinic Acetylcholine Receptor signaling pathway were also enriched in all compared groups (Table 2). De novo pyrimidine deoxyribonucleotide biosynthesis, Pyrimidine Metabolism, 2-arachidonoylglycerol biosynthesis, Cholesterol biosynthesis, Gamma-aminobutyric acid synthesis, Aminobutyrate degradation and Histamine synthesis pathways were observed in only active TB vs no TB comparison. Fructose galactose metabolism, Heme biosynthesis, Pentose phosphate pathway, Pyridoxal-5-phosphate biosynthesis, S-adenosylmethionine biosynthesis, Leucine biosynthesis, Isoleucine biosynthesis, Alanine biosynthesis, Valine biosynthesis, Anandamide and degradation were also entirely specific to the latent TB vs. no TB comparison.

DisGeNET (disease associations)

Out of 274 queried terms, only lymphoid leukemia remained significant after multiple-testing correction (Benjamini–Hochberg FDR = 0.0119) for the prior TB vs. no prior TB dmDNA patterns. Specifically, 139 of 213 DisGeNET-annotated lymphoid leukemia genes overlapped with our list (overlap = 139/213), yielding an odds ratio of 1.96 and a combined score of 26.7.

dbGaP (GWAS phenotypes)

In contrast, enrichment against the dbGaP library identified seven phenotype associations at FDR < 0.05 (out of ~ 300 tested) for the prior-TB vs. no-prior-TB DNAm patterns. These were lymphocytes (overlap = 36/44; OR = 4.69; FDR = 0.0025; combined score = 55.4), tunica media (71/104; OR = 1.58; FDR = 0.0095; combined

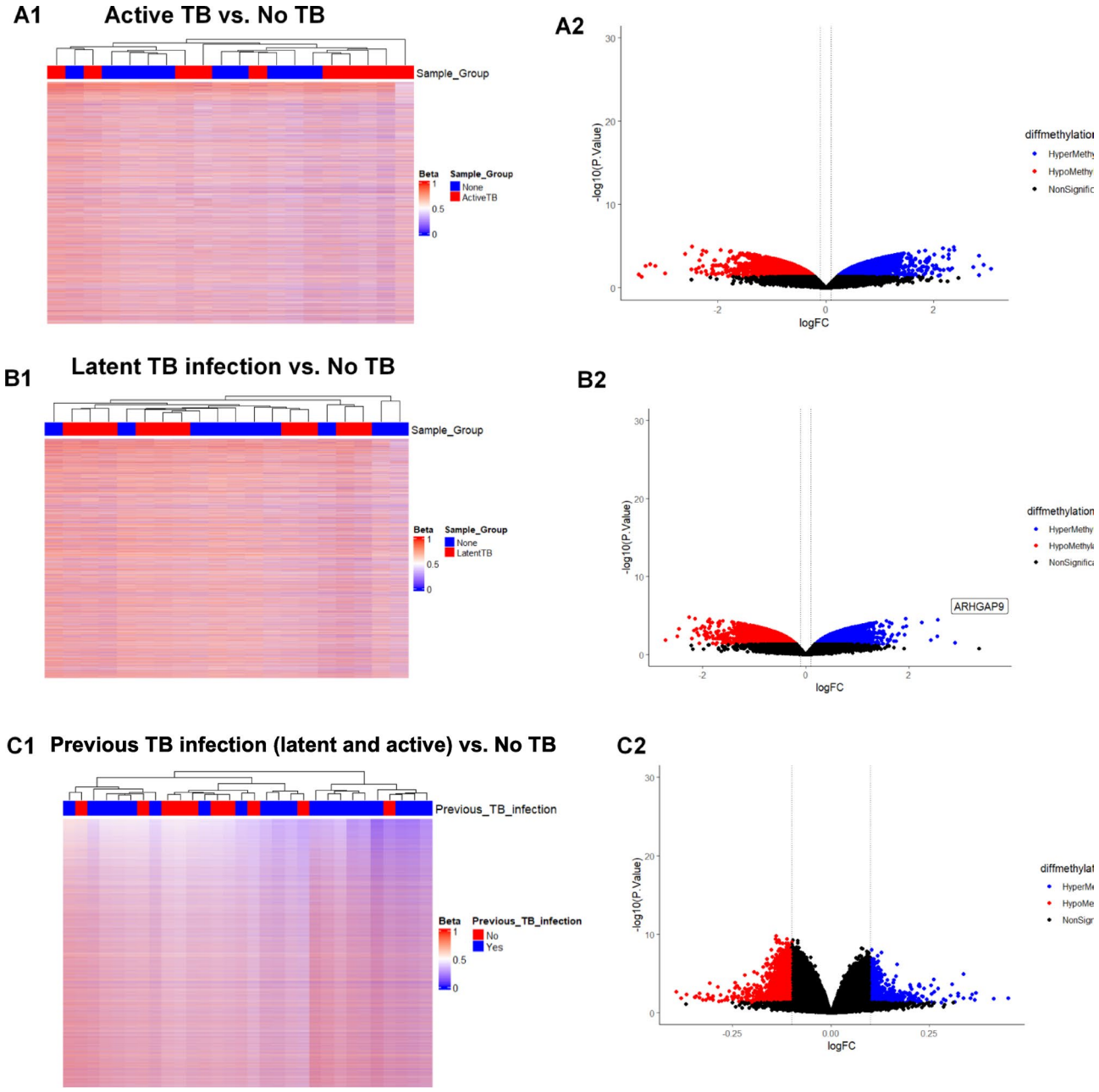

Fig. 2. Heatmaps A1, B1, and C1 derived from beta values of the differently methylated CpG sites identified in pairwise comparison across different groups with stringency criteria of adjusted $P < 0.01$ and mean methylation difference > 0.2. Dendrogram shows separation based on groups. Enhanced Volcano plots A2, B2 and C2 of the differentially methylated CpG-sites separating different groups. The x-axis is the log fold change and the y-axis is the negative $\log_{10}$ of *p*-value with the cut off p-value of 0.05 shown with dash-dotted horizontal line. Blue CpGs indicate hypomethylated while red CpGs are hypermethylated.

score = 17.4), asthma (72/107; OR = 2.10; FDR = 0.0096; combined score = 18.6), body mass index (253/437; OR = 1.22; FDR = 0.0096; combined score = 16.8), leukocyte count (31/40; OR = 3.14; FDR = 0.0149; combined score = 15.2), body height (198/294; OR = 1.31; FDR = 0.0231; combined score = 12.5), body composition (154/241; OR = 1.28; FDR = 0.0314; combined score = 11.2), and cell adhesion molecules (89/135; OR = 1.45; FDR = 0.0427; combined score = 9.8).

Discussion

In this study, we investigated DNAm patterns associated with prior TB infection in individuals with HIV. Our findings reveal that both prior TB infection (LTBI or active TB) are associated with distinct DNAm changes, particularly in pathways related to neural function and development, cardiovascular health, and cancer risk. In addition, there was significant epigenetic age acceleration in PWH, which was notably more pronounced

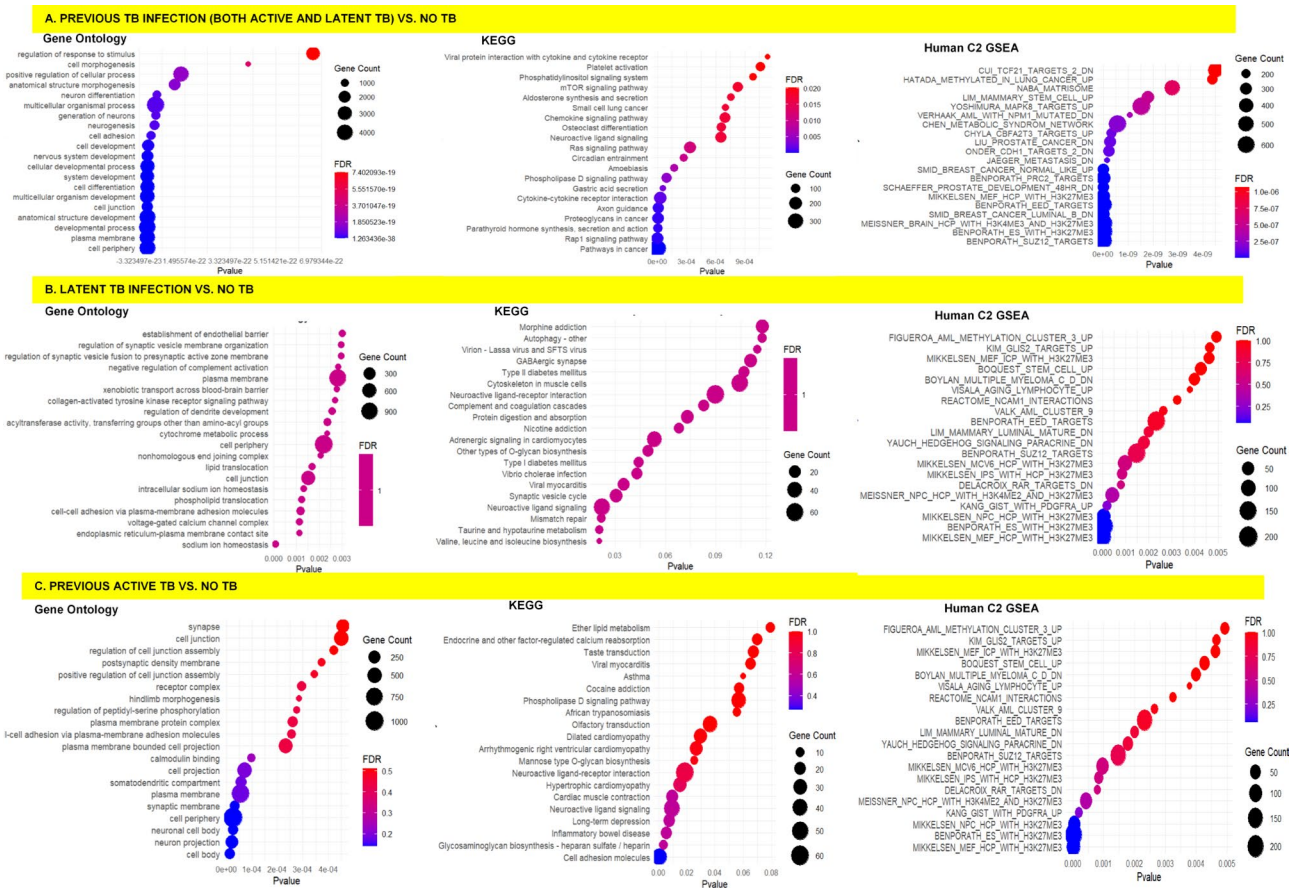

Fig. 3. Pathway enrichment analysis for association of prior TB and DNA methylation. In each panel, the x-axis represents the *p*-value for pathway enrichment (adjusted for false discovery rate [FDR]), while the y-axis lists the enriched pathways. The size of the dots indicates the number of genes involved in each pathway, and the color gradient represents the FDR, with red indicating higher significance (lower FDR).

in individuals with a history of TB infection (21.60 years of acceleration) compared to TB-naïve individuals (17.42 years of acceleration). This suggests that prior TB may exacerbate the already accelerated aging processes driven by HIV, potentially contributing to an earlier onset of age-related comorbidities. Similar to our findings, Bobak et al. (2022) demonstrated that active tuberculosis in humans was associated with approximately 12.7 years of cellular aging, as measured by epigenetic clocks (Horvath clock), and 14.38 years using gene expression-based cellular clocks[23].

The analysis of DNAm patterns identified a substantial number of dmCpGs and DMRs. Specifically, 7461 dmCpGs (150 DMRs) were found when comparing prior active TB to no TB, and 8598 dmCpGs (39 DMRs) when comparing LTBI to no TB. The combined group of any prior TB showed 71,774 dmCpGs (14 DMRs) compared to no TB. These findings highlight that both forms of prior TB exposure leave lasting epigenetic marks. Genes implicated in these DMRs, such as *GRAMD1C* and *DPP6* in active TB history, and *PLEKHG5* and *STK32C* in LTBI history, warrant further investigation for their roles in TB-related sequelae. For instance, genes like *KCNN3* and *BRSK2* were identified in the overall prior TB group, pointing to common epigenetic alterations irrespective of TB history type.

Functional enrichment analyses of these DNAm changes provide insights into the potential biological consequences. Across individuals with any prior TB exposure, pathways related to neural functions such as neurogenesis, neuron differentiation, axon guidance, and neuroactive ligand signaling were significantly enriched. This aligns with reports of increased risk for neurological and cognitive disorders post-TB and suggests an epigenetic basis for these observations in PWH[12,13,35,36]. The consistent enrichment of the Wnt signaling pathway across different TB comparison groups, as identified through PANTHER database analysis, further supports a role for altered neurodevelopmental and cell signaling processes[37].

Cardiovascular health also appears to be epigenetically impacted by prior TB. Pathways including platelet activation and vascular smooth muscle contraction were enriched in the combined prior TB group. The dbGaP analysis further identified an association between prior TB DNAm patterns and "tunica media", a component of blood vessels, suggesting structural or functional vascular changes that could engender atherosclerosis in the setting of hyperlipemia[38]. These findings support epidemiological evidence linking TB to increased cardiovascular disease risk[19]. The enrichment in pathways related to cholesterol biosynthesis and body mass index suggests epigenetic changes underlying the effect that prior TB migt have on cardiovascular disease risk

Pathway	Prior TB (both active and latent) vs. No TB	Active TB vs. No TB	Latent TB vs. No TB	Active TB vs. Latent TB
Wnt signaling pathway	159 genes	38 genes	41 genes	60 genes
Inflammation mediated by chemokine and cytokine signaling	123 genes	23 genes	26 genes	22 genes
Integrin signaling pathway	85 genes	25 genes	22 genes	25 genes
Angiogenesis	102 genes	19 genes	20 genes	18 gene
Cadherin signaling pathway	100 genes	19 genes	22 genes	45 genes
Heterotrimeric G-protein signaling pathway	73 genes	20 genes	15 genes	22 genes
FGF signaling pathway	59 genes	17 genes	12 genes	9 genes
Nicotinic Acetylcholine Receptor signaling pathway	44 genes	13 genes	9 genes	14 genes
EGF signaling pathway	70 genes	14 genes	7 genes	12 genes
CCKR signaling pathway	87 genes	19 genes	17 genes	21 gene
Interleukin signaling pathway	43 genes	7 genes	11 genes	8 genes
Apoptosis signaling pathway	53 genes	18 genes	11 genes	16 genes
PDGF signaling pathway	66 genes	12 genes	13 genes	16 gene
T-cell Activation	42 genes	5 genes	5 genes	9 genes
Hedgehog signaling pathway	11 genes	3 genes	3 genes	2 genes
Pyrimidine Metabolism	6 genes	3 genes	None	None
Fructose galactose metabolism	7 genes	None	2 genes	None
Histamine synthesis	None	1 gene	None	None
mRNA splicing	None	None	None	1 gene
Thiamin metabolism	None	None	None	1 gene

Table 2. Pathway enrichment in PWH with and without prior TB from the PANTHER database.

factors as we have previously reported in our study setting[39]. Our previous work among PWH with prior active TB suggests that prior TB is associated with normal body mass index and a lower prevalence of dyslipidemia[39].

The study also uncovered significant DNAm alterations in cancer-related pathways. In individuals with any prior TB, enrichment was seen for pathways associated with small cell lung cancer, prostate cancer, lung cancer, breast cancer, hepatocellular carcinoma, thyroid cancer, and proteoglycans in cancer. Furthermore, DisGeNET analysis directly linked DNAm patterns in the prior TB group to lymphoid leukemia. These epigenetic changes may contribute to the increased cancer incidence observed in TB survivors particularly for lung, hepatobiliary, and leukemia[15,40]. Moreover, the prominent enrichment in the gene sets associated with embryonic stem cell identity: BENPORATH_ES_WITH_H3K27ME3, BENPORATH_EED_TARGETS, and BENPORATH_SUZ12_TARGETS suggests potential risk for the development of aggressive, poorly differentiated, and estrogen receptor-negative tumors[41]. The association between prior TB and advanced cancer types needs to be demonstrated in epidemiological studies.

The mechanisms underlying the observed DNAm changes and their specific contributions to long-term complications warrant further investigation by larger studies. Inflammation-related pathways, including chemokine and cytokine signaling and integrin signaling, were consistently enriched across comparisons, underscoring the role of persistent inflammation in driving these epigenetic modifications and subsequent long-term complications[22]. The association of prior TB DNAm with phenotypes such as asthma and altered leukocyte counts in the dbGaP analysis further supports ongoing immune dysregulation. Additionally, the interaction between TB infection, HIV infection, and antiretroviral therapy may further contribute to epigenetic changes and long-term complications.

Our findings of widespread and persistent DNA methylation changes in PWH with a history of prior TB complement and extend studies like DiNardo et al. (2020), which demonstrated DNA hypermethylation in critical immune pathways leading to dampened host immune responsiveness during active TB, with some changes persisting post-treatment[42]. While DiNardo et al. focused on hypermethylation as a mechanism for immediate immune evasion by *M. tuberculosis*, our study reveals a more complex, long-term epigenetic landscape in PWH following prior TB, characterized by both hyper- and hypomethylation across a broader range of pathways implicated in neural function, cardiovascular health, cancer, and accelerated aging. Thus, while active infection may induce specific hypermethylation to suppress acute immunity, our results suggest that prior TB exposure in PWH leads to a distinct and more diverse set of lasting epigenetic modifications that likely contribute to the increased risk of chronic comorbidities and accelerated biological aging observed in this population.

Our study has several limitations. The cross-sectional design limits our ability to establish causal relationships between prior TB infection and DNAm changes. The relatively small sample size may have limited our power to detect additional significant associations. Furthermore, our study focused on peripheral blood DNAm, which may not fully reflect epigenetic changes in other tissues relevant to long-term complications. The use of the IGRA to identify LTBI can misclassify PWH due to immune anergy. However, PWH in our study were mostly immune competent (median CD4 count of > 900 cells), making the risk of misclassification low.

Conclusion

In conclusion, prior TB infection in PWH is associated with extensive DNA methylation alterations and pronounced epigenetic age acceleration in pathways linked to neurocognitive health, cardiovascular function, and cancer susceptibility. These epigenetic signatures offer promising biomarkers for identifying PWH at highest risk of TB-related long-term comorbidities. Longitudinal studies are warranted to validate these methylation patterns as predictive markers and to explore targeted interventions—such as anti-inflammatory therapies or epigenetic modulators—to mitigate post-TB sequelae in this vulnerable population.

Data availability

Datasets used in this study are available from https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE304107.

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Author contributions
JBB—conceptualization, methodology, investigation, data accrual, formal analysis, interpretation of results, drafting manuscript, editing manuscript, final approval. SN—investigation, data accrual, interpretation of results, drafting manuscript, editing manuscript, final approval. DK—investigation, data accrual, interpretation of results, drafting manuscript, editing manuscript, final approval. BN—investigation, data accrual, interpretation of results, drafting manuscript, editing manuscript, final approval. HK—methodology, investigation, data accrual, formal analysis, interpretation of results, drafting manuscript, editing manuscript, final approval. IJ—interpretation of results, drafting manuscript, editing manuscript, final approval. WG—methodology, investigation, formal analysis, interpretation of results, drafting manuscript, editing manuscript, final approval. NN—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. NB—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. EW—investigation, data accrual, interpretation of results, drafting manuscript, editing manuscript, final approval. JR—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. NJ—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. CK—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. MS—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. AC—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. IN—methodology, investigation, data accrual, formal analysis, interpretation of results, drafting manuscript, editing manuscript, final approval. SM—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. SG—investigation, interpretation of results, drafting manuscript, editing manuscript, final approval. BK—investigation, dinterpretation of results, drafting manuscript, editing manuscript, final approval.

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Competing interests
The authors declare that they have no relevant competing interests.

Consent for publication
Not applicable.

Ethical approval
Study participants provided written informed consent before study measurements were undertaken. The study protocol was approved by the Mildmay Uganda Research and Ethics Committee (#REC REF MUREC-107-2022). Further, the Uganda National Council of Science and Technology provided additional approval as required by the guidelines for conducting research in Uganda (HS2328ES). All methods were performed in accordance with the relevant guidelines and regulations.

Additional information
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