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RESEARCH ARTICLE

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Dissecting epigenetic age acceleration in amyotrophic lateral sclerosis

Polina Merbaum [a], Ramona Zwamborn [a], Paul Hop [a], Project MinE ALS Sequencing Consortium[b], Wouter van Rheenen [a] and Jan Veldink [a]

[a]Department of Neurology, UMC Utrecht Brain Center, Utrecht University, Utrecht, The Netherlands; [b]Project MinE Sequencing Consortium, Utrecht, The Netherlands

ABSTRACT

Aim: We compared signatures of epigenetic aging in amyotrophic lateral sclerosis (ALS) patients and healthy controls to investigate the role of potential confounders and genetic subgroups.

Methods: We used whole-blood methylome profiles for 5,146 ALS patients and 2,156 controls available for Project MinE. We predicted biological age with three generations of epigenetic clocks and estimated age acceleration by regressing our model on control individuals to evaluate case/control differences. To investigate the contribution of *C9orf72* expansions, we regressed the model on *C9orf72*-negative ALS patients. The predicted DunedinPACE pace of aging and telomere length additionally characterized aging dynamics.

Results: We found that white blood cell type proportions confound the previously observed increase in the pace of biological aging in ALS. When correcting for cell counts, there is no evidence for accelerated epigenetic aging compared to controls, except for ALS patients with the *C9orf72* repeat expansion. None of the epigenetic age acceleration scores contributed to survival.

Conclusion: Our study revealed no significant difference in the pace of biological agingbetween ALS patients and controls, except for ALS patients carrying the *C9orf72* mutation. We emphasize the importance of altered white blood cell proportions in general ALS pathophysiology as opposed to accelerated aging per se.

PLAIN LANGUAGE SUMMARY

Amyotrophic Lateral Sclerosis (ALS) is a serious disease that affects the nervous system and makes it hard for people to move. One of the main risk factors for developing ALS is getting older. Our study looked at whether people with ALS have signs of aging faster than those who don't have the disease. To do so, we examined changes in chemical markers on DNA, called DNA methylation, that occur as people age. We studied blood samples from 5,146 ALS patients and 2,156 healthy individuals, using several tools that estimate biological age and how quickly it changes. Our results showed that differences in immune cell composition help explain why it seems like ALS patients age faster. After taking these differences into account, we found that, generally, ALS patients did not age faster than healthy controls. However, those with the *C9orf72* genetic mutation did show signs of aging more quickly. Overall, our findings suggest that changes in immune cells are very important in ALS and may be more significant than the idea of accelerated aging, except for patients with the *C9orf72* mutation.

ARTICLE HISTORY

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Epigenetics; epigenetic clock; ALS; age acceleration; neurodegeneration; C9orf72 repeat expansion; epigenetics and disease

1. Introduction

Amyotrophic Lateral Sclerosis (ALS) is a devastating neurodegenerative disease, characterized by progressive motor deficit and eventual death [1]. In ALS, both upper and lower motor neurons are affected, which leads to muscle weakness and atrophy. ALS is known for its rapid progression, with a median survival time between 20 and 48 months after the disease onset [2]. Aging is one of the most significant risk factors for ALS, as well as male sex, smoking, and familial history of the disease [3]. Additionally, pathophysiological hallmarks of ALS closely resemble those associated with aging, such as cellular senescence, DNA damage, and neuroinflammation [4]. However, the origin of these changes is not completely understood.

One hypothesis suggests that these changes may result from an increased pace of biological aging. Biological age is a measure of an individual's age based on age-dependent biological changes. It offers a more accurate reflection of internal processes and cumulative exposures than chronological age, providing better insights into an individual's mortality risk and general healthm [5]. It can be measured with various polymethylation scores addressed as 'epigenetic clocks,' which assess different biological age indicators.

Some epigenetic clocks, like the Zhang clock [6], were designed to accurately predict chronological age, while others, like GrimAge or PhenoAge [7,8], specifically predict all-cause mortality. Additional clocks measure aspects of cellular aging, such as telomere length (DNAmTL) or stem cell division rate

CONTACT Jan Veldink j.h.veldink@umcutrecht.nl Department of Neurology, UMC Utrecht Brain Center, Utrecht University, F02.131, 85500, Utrecht 3508 GA, The Netherlands

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Article highlights

- Epigenetic age acceleration scores do not differ between the general ALS population and controls after adjusting for WBC proportions;
- Increased pace of aging is not a universal feature of ALS but takes place in a subgroup of patients with *C9orf72* expansion;
- Previously reported age acceleration in general ALS largely reflects shifts in the proportion of immune cells;
- None of the epigenetic age acceleration scores contributed to survival;
- White blood cell proportion is an important covariate in epigenetic research.

[9,10]. These epigenetic clocks help determine whether an individual has accumulated more or fewer age-related changes than would be expected based on their chronological age, reflecting their cumulative aging burden.

Another notable epigenetic clock is the DunedinPACE [11]. It was trained on longitudinal data and measures the pace of aging at the time of sampling, independent of chronological age.

Epigenetic clocks are commonly used to mathematically determine the pace of biological aging. When biological aging exceeds chronological aging, it is called epigenetic age acceleration (EAA). EAA is reported in years of accumulated aging and is associated with an increased overall mortality rate and development of various conditions, including type 2 diabetes, stroke, and cancer [12]. Given that aging is a significant risk factor for neurodegeneration, EAA has been observed in neurodegenerative and psychiatric diseases, such as Alzheimer's disease, Parkinson's disease, and schizophrenia [13,14]. Similarly, the aging process is of great interest in ALS. For instance, aging neurons accumulate TDP-43 in the cytoplasm, where it can form toxic aggregates, the pathological hallmark of ALS [15]. Furthermore, EAA has been linked to an earlier onset and shorter survival in ALS [16,17].

We analyzed the largest methylation dataset in ALS to date, including 5,146 patients and 2,156 controls [18]. Then, we compared age acceleration rates between ALS patients and controls to evaluate the role of age acceleration in ALS susceptibility and survival. We selected five epigenetic clocks from different categories – the first-generation Horvath clock [19], PCGrimAGe, the principal component-based derivative of second-generation mortality predictor GrimAge [7,20], and the third-generation pan-mammalian clock [21], for which we estimated age acceleration in years for each individual. We also predicted the pace of aging with DunedinPACE [11] and leucocyte telomere length with DNAmTL [9]. Ultimately, we compared epigenetic age acceleration between ALS patients with *C9orf72* repeat expansion and those without the expansion.

2. Methods

2.1. Study population and ethics

We used DNA methylation beta-values for 7,302 individuals (5,146 patients with ALS and 2,156 controls) from ProjectMinE, quantified with the Illumina HumanMethylation450k array (MinE 450k, $N = 4,425$) and Illumina HumanMethylationEPIC array (MinE EPIC, $N = 2,877$). MinE 450k and MinE EPIC cohorts, as well as the DNA methylation quality control procedure, are described in the manuscript by Hop et al. in the 'Study Design' section of Methods [22]. Our sample size was determined by the availability of high-quality DNA methylation data and phenotype information. Although we did not perform formal a priori power calculations, the size of our cohort provides strong statistical power to detect modest epigenetic effects while accounting for multiple covariates. Individuals with specific ALS subtypes, including progressive muscular atrophy, primary lateral sclerosis, or mixed presentations, were excluded from the study.

Survival analysis was performed on a subset of ALS individuals ($N = 5,022$) with known survival time and survival status (dead or alive) at the time of the analysis.

All participants gave written informed consent, and the relevant local institutional review boards approved this study. Detailed information can be found in the manuscript by van Rheenen et al. in the Supplementary Information section [23].

2.2. Epigenetic age acceleration estimation

We used five EAA parameters for our analysis, all calculated with the *dnaMathyAge* package (v.0.2.0) [24]. First, we predicted age with the first-generation Horvath epigenetic clock [19], PCGrimAge, a principal component-based derivative of the second-generation mortality predictor GrimAge epigenetic clock [7,20], and the third-generation pan-mammalian clock [21], which derives a relative cellular age. GrimAge estimates epigenetic mortality risk using DNA methylation surrogates of plasma proteins and smoking, while PCGrimAge applies principal component analysis to enhance robustness and predictive performance [20]. Then, for the case/control study, we calculated EAA for these clocks by taking the residuals from a Loess regression model, in which DNA methylation age was regressed against chronological age for control individuals as a baseline. This approach ensures that age acceleration reflects deviations from the expected epigenetic age in healthy controls, providing a reference point for comparing cases. A positive EAA means that a person's epigenetic age is higher than one expected for a healthy person of the same chronological age. For *C9orf72*-positive vs. *C9orf72*-negative analysis, we calculated EAA by taking the residuals from a Loess regression model, in which DNA methylation age was regressed against chronological age for *C9orf72*-negative ALS patients. This approach provided a disease-specific baseline for age acceleration, against which *C9orf72*-positive individuals could be compared. Loess regression captured the non-linear patterns of aging across various age groups, generating fitted biological age values [24]. This method is often preferred for large datasets because it is less sensitive to noise [25]. Second, we estimated the pace of aging with DunedinPACE [11] and the leukocyte telomere length predictor DNAmTL [9]. DunedinPACE > 1 and shorter telomere length was considered an increase in the pace of aging.

2.3. Covariate estimation, model selection, and association analyses

For 1,254 individuals with missing age at the date of blood draw, we imputed chronological age with the Zhang

epigenetic clock [6]. We compared actual chronological age with imputed age for the other 6,048 samples and calculated their correlation coefficient. To assess whether results were affected by imputed age, we performed an analysis on non-imputed subsets only ($N = 6,048$ for case/control status and 4,082 for *C9orf72* status). Detailed results are provided in the Supplementary Materials (see Sensitivity analysis). Smoking score and smoking status ('Current smoker,' 'Former smoker,' or 'Never Smoked') were predicted with the *EpiSmoker* R package [26]. The composition of white blood cells (WBC) was predicted using the *FlowSorted.BloodExtended.EPIC* R package [27], with its modified 450k version tailored to match the array type. We compared clinically defined WBC proportions available for 861 Dutch individuals from MinE 450k with those predicted with *FlowSorted*, and calculated their correlation coefficient. The clinical WBC proportion was measured within 4 months of DNA methylation blood withdrawal. Since WBC composition was expressed as a proportion of all 12 cell types, we excluded the most abundant and variable type, neutrophils, to prevent collinearity in the model. To assess whether results were affected by cell type selection, we performed a sensitivity analysis, replacing the least variable cell type, CD8+ native cells, with neutrophils. Detailed results are provided in the Supplementary Materials (see Sensitivity analysis).

First, we performed a logistic regression analysis for each cohort between the estimated age acceleration parameters and case/control status, adjusting for chronological age, sex, predicted smoking status, and experimental batch. We compared the fit of this model with the model that included the proportion of white blood cells as a covariate using the Akaike information criterion (AIC) [28], where lower AIC values indicate a better fit – an explanation of the outcome variation. Since the AIC values did not follow a normal distribution, we used the Wilcoxon signed-rank test, a non-parametric method that is appropriate for paired comparisons without assuming normality. A p-value < 0.05 was deemed statistically significant, indicating a meaningful difference in model fit.

Second, we performed a logistic regression analysis for each cohort between the estimated age acceleration parameters and *C9orf72* expansion status for ALS patients alone, adjusting for chronological age, sex, predicted smoking status, experimental batch, site of disease onset, and *C9orf72* expansion status. The *C9orf72* expansion status was determined with PCR or ExpansionHunter [29].

Then, we performed survival Cox proportional hazard regression analysis in ALS cases with known survival status, integrating the epigenetic age acceleration parameters and biological and technical parameters listed above as covariates.

To conclude the significance of the association between the EAA and investigated covariates, we combined results with inverse variance weighted (IVW) fixed-effects meta-analysis, as implemented in the *meta* R package (v.4.19–2) [30]. P-values were adjusted for multiple testing with the Benjamini-Hochberg algorithm. P-values < 0.05 were considered statistically significant to support evidence of association.

3. Results

This study used data from 5,146 patients with ALS and 2,156 controls from the ProjectMinE consortium [22]. Data consisted of two cohorts that differ by the sequencing array – Illumina HumanMethylation450k and HumanMethylationEPIC. Detailed phenotype information is listed in Table 1.

We imputed the chronological age for 1,254 individuals who had missing age data at the time of blood draw using the Zhang epigenetic clock. Among the 6,048 individuals with known chronological ages, we found a strong correlation between chronological age and the age predicted by the Zhang clock, with an R^2 value of 0.915 (CI = [0.910; 0.919], $p < 2 \times 10^{-16}$), which was the strongest among all clocks (Horvath $R^2 = 0.77$, CI = [0.76; 0.78], $p < 2 \times 10^{-16}$; PCGrimAge $R^2 = 0.87$, CI = [0.86; 0.88], $p < 2 \times 10^{-16}$; pan-mammalian $R^2 = 0.04$, CI = [0.03; 0.05], $p < 2 \times 10^{-16}$).

Additionally, we investigated the correlation between clinically defined WBC proportions and the epigenetically predicted WBC proportions available for 861 ALS individuals from MinE 450. The correlation yielded the following R^2 : neutrophils = 0.74, CI = [0.71; 0.76], $p < 2 \times 10^{-16}$; lymphocytes = 0.80, CI = [0.78; 0.82], $p < 2 \times 10^{-16}$, monocytes = 0.62, CI = [0.58; 0.66], $p < 2 \times 10^{-16}$; eosinophis = 0.44, CI = [0.39; 0.49], $p < 2 \times 10^{-16}$, and basophils = 0.02, CI = [0.009; 0.05], $p = 1.18 \times 10^{-6}$.

3.1. No association between ALS case/control status and age acceleration

In the model selection phase, we first assessed the relationship between EAA and chronological age for all clocks and observed significant correlations for DNAmTL and DunedinPACE in both ALS and controls and in PCGrimAge for ALS cases only (see Supplementary Figure S1 and Table S1). Model comparison with ANOVA indicated that for these clocks, models including chronological age explained outcome variation significantly better than models without age, underscoring the importance of including chronological age as a covariate (DNAmTL, $p < 1 \times 10^{-308}$; DunedinPACE, $p < 1 \times 10^{-308}$; PCGrimAge, $p < 1 \times 10^{-308}$). Although this was not the case for the other clocks (Horvath, $p = 0.32$; pan-mammalian, $p = 0.45$), for which models with and without chronological age did not differ significantly, we included chronological age in all models for consistency.

Then, we applied the model correcting only for sex, age, experimental batch, and smoking score, and consistently observed a signal for accelerated aging in patients with ALS compared to controls when using PCGrimAge (BETA = 0.27, SE = 0.018, $p = 2.60 \times 10^{-50}$), pan-mammalian clock (BETA = 0.09, SE = 0.02, $p = 1.06 \times 10^{-3}$), DunedinPACE (BETA = 0.13, SE = 0.02, $p = 5.96 - \times 10^{-8}$), and DNAmTL (BETA = –0.05, SE = 0.01, $p = 2.63 \times 10^{-3}$), but not when using Horvath clock (BETA = –0.001, SE = 0.02, $p = 0.951$) (Figure 1(A)). A positive effect indicated increased biological aging for all EAA parameters, whereas for DNAmTL, the negative effect reflected shorter telomere length, which is also consistent with accelerated aging.

We then determined the WBC proportion from DNA methylation values using the *FlowSorted* R package. When we corrected for WBC proportion by including it as a covariate in the regression model, we observed no evidence for epigenetic age acceleration in ALS (PCGrimAge: BETA = 0.007, SE = 0.01, $p =$

Table 1. Demographic and clinical characteristics of the study population population with detailed information per case/control status and study cohort.

	ALS		Controls	
	MinE 450k	MinE EPIC	MinE 450k	MinE EPIC
N	2989	2157	1436	720
Sex (Female%)	1204 (40%)	863 (40%)	640 (44%)	338 (54%)
Age (years)				
Mean (±SD)	62.9 (±11.4)	62.8 (±11.9)	61.8 (±10.7)	61.8 (±11.7)
Missing (%)	405 (13%)	659 (30%)	18 (1%)	172 (23%)
Predicted Age (years) with epigenetic clocks				
Zhang	63.8 (±10.4)	61.4 (±11.6)	62.5 (±9.8)	60.7 (±11.2)
Horvath	63.0 (±10.5)	62.8 (±9.5)	61.8 (±9.9)	62.1 (±9.2)
PCGrimAge mortality predictor	75.0 (±9.4)	74.7 (±9.7)	73.1 (±8.9)	73.0 (±9.3)
Pan-mammalian relative age	44.4 (±21.3)	33.0 (±20.6)	41.9 (±20.2)	31.2 (±17.8)
DunedinPACE (units)	1.07 (±0.123)	1.06 (±0.126)	1.04 (±0.120)	1.05 (±0.128)
DNAmTL (kb)	6.92 (±0.244)	7.01 (±0.246)	6.96 (±0.246)	7.03 (±0.247)
Site of Onset				
Spinal	1943 (65%)	1453 (67%)	–	–
Bulbar	840 (28%)	548 (25%)	–	–
Generalized	98 (3%)	77 (4%)	–	–
Thoracic	64 (2%)	40 (2%)	–	–
Missing	44 (2%)	39 (2%)	–	–
Survival status			-	–
Deceased	2527 (85%)	1581 (73%)	–	–
Alive	425 (14%)	558 (26%)	–	–
Missing	37 (1%)	18 (1%)	–	–
Survival (months)*			-	–
Median (Q1-Q3)	34.5 (21.9–58.4)	36.5 (23.8–65.5)	–	–
Missing	57 (2%)	52 (2%)	–	–
***C9orf72* status**			-	–
Expanded (≥30 repeats)	188 (6.2%)	129 (5.9%)	–	–
Normal	2770 (92%)	2003 (93%)	–	–
Missing	31 (1%)	25 (1%)	–	–

*Survival median and interquartile range were estimated from Kaplan-Meier survival curves, accounting for censoring.
Abbreviations: ALS, amyotrophic lateral sclerosis; kb, kilobase.

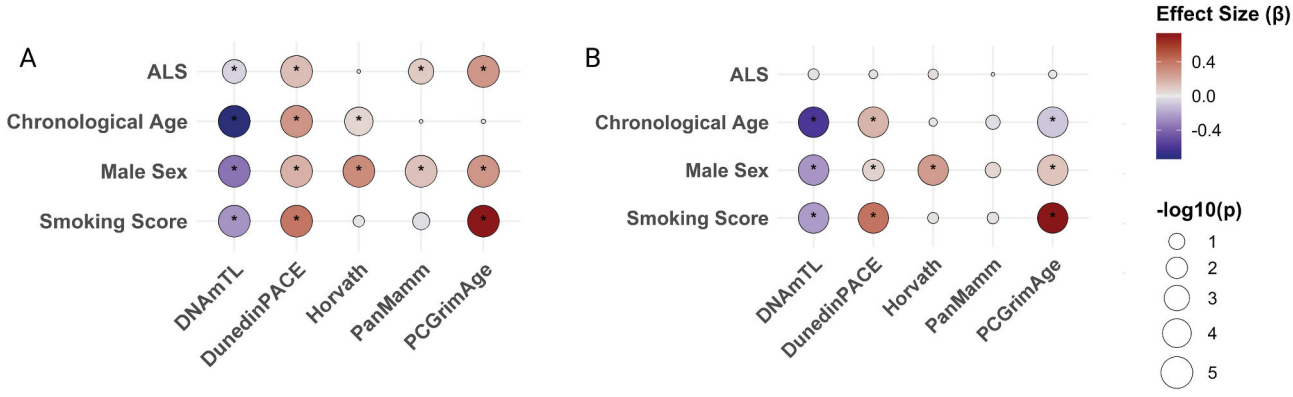

Figure 1. Effect sizes and significance of covariates across models without (A) and with white blood cell (WBC) adjustment (B). Each point represents a covariate within a model, with fill color indicating the estimated fixed effect size (β): red for positive, blue for negative, and gray near zero. A positive effect indicates increased biological aging for all epigenetic age acceleration (EAA) parameters, whereas for DNAmTL, the negative effect reflects shorter telomere length, which is also consistent with accelerated aging. Effect sizes are normalized for comparability across all parameters in the model. Point size corresponds to statistical significance, represented as $-\log_{10}$(multiple testing adjusted p) values capped at 5 to facilitate visualization. Asterisks denote covariates with adjusted $p < 0.05$. Covariates include case/control phenotype, chronological age, sex, batch, and smoking score, and (for B), white blood cell proportion.

0.66; Horvath clock: BETA = 0.02, SE = 0.02, $p = 0.47$; pan-mammalian clock: BETA = 0.002, SE = 0.02, $p = 0.97$; DunedinPACE: BETA = −0.01, SE = 0.02, $p = 0.60$; DNAmTL: BETA = 0.01, SE = 0.01, $p = 0.39$) (Figure 1(B)). (A detailed report of the results per sequencing cohort can be found in Supplementary Material, Supplementary Table S2). The model that included WBC proportions demonstrated a significantly better fit than the one that did not, as shown by the Wilcoxon test comparing the Akaike Information Criterion (AIC) for all epigenetic clocks. ($p = 0.03$). Thus, this adjusted model was used for all subsequent analyses. Our sensitivity analysis showed that these results were not affected by samples with imputed ages or changes in the selection of cell types as covariates in the model (see Supplementary Materials for the Sensitivity analysis).

Across all epigenetic clocks, except pan-mammalian EAA, male sex was associated with age acceleration ($p < 0.01$, see Supplementary Table S3). Higher smoking score was also strongly associated with age acceleration in PCGrimAge, DunedinPACE, and DNAmTL ($p < 0.0001$, see Supplementary Tables S4).

3.2. WBC is an important covariate in age acceleration studies

To explore which white blood cell types were associated with which epigenetic age acceleration parameter, we performed a linear regression between EAA parameters and WBC proportion, adjusting for age, sex, experimental batch, and smoking score. We found that each EAA parameter was significantly associated with at least six out of twelve white blood cell types studied, underscoring the importance of correcting for WBC in any EAA analysis (Figure 2, Supplementary Table S5). Notably, a higher proportion of CD4+ T cells and natural killer cells was linked to a reduction in biological aging across all five EAA parameters, while a higher proportion of basophils was associated with an increase in biological aging.

Additionally, we examined whether the CpG sites used to estimate WBC proportions overlapped with those used in the model of each epigenetic clock. PCGrimAge demonstrated the greatest overlap for both the 450k and EPIC arrays, while the other clocks exhibited minimal to no overlap (see Table 2). Moreover, we assessed the 514 CpG sites involved in the construction of the Zhang clock to determine if any were used in WBC prediction. We found no overlap with the *FlowSorted.BloodExtended.EPIC* array, but there were 7 overlapping CpG sites with the *FlowSorted.BloodExtended.450k* array.

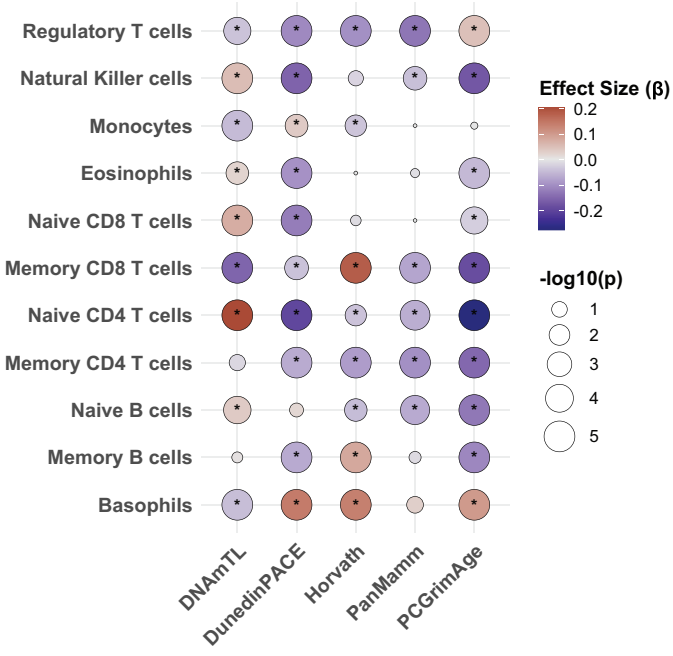

Figure 2. Associations between EAA parameters and white blood cell proportions in multivariate linear regression models. Each point represents a covariate within a model, with fill color indicating the estimated fixed effect size (β): red for positive, blue for negative, and gray near zero. A positive effect indicates increased biological aging for all EAA parameters, whereas for DNAmTL, the negative effect reflects shorter telomere length, which is also consistent with accelerated aging. Effect sizes are normalized for comparability across all parameters in the model. Point size corresponds to statistical significance, represented as $-\log_{10}$(multiple testing adjusted p) values capped at 5 to facilitate visualization. Asterisks denote covariates with adjusted $p < 0.05$. Covariates include chronological age, sex, batch, and smoking score.

3.3. Increased pace of aging takes place in the C9orf72-positive ALS subgroup

In the second phase, we investigated the relationship between the EAA and *C9orf72* expansion status in ALS individuals, comparing those with and without the expansion. We observed that *C9orf72* expansion was associated with increased DunedinPACE pace of aging and shorter telomeres measured with DNAmTL compared to ALS patients with a non-expanded repeat (PCGrimAge: BETA = −0.018, SE = 0.02, $p = 0.61$; Horvath clock: BETA = −0.001, SE = 0.02, $p = 0.94$; pan-mammalian clock: BETA = 0.029, SE = 0.06, $p = 0.71$; DunedinPACE: BETA = 0.156, SE = 0.05, $p = 5.3 \times 10^{-3}$; DNAmTL: BETA = −0.110, SE = 0.03, $p = 3.3 \times 10^{-3}$) (Figure 3). Our sensitivity analysis revealed that these results were not affected by samples with imputed ages or changes in the selection of cell types as covariates in the model (see Supplementary Materials for the Sensitivity analysis).

3.4. None of the epigenetic age acceleration scores contributed to survival

Finally, we performed a Cox proportional hazards survival analysis on the subset of ALS individuals ($N = 5{,}022$) for whom we had known survival time and status. We tested all epigenetic aging predictors and adjusted for sex, age, experimental batch, smoking status, WBC, site of disease onset, and *C9orf72* expansion. We observed no effect of EAA parameters on ALS survival. As has been previously reported [31,32], we observed an association between shorter survival and older chronological age (HR = 1.20, SE = 0.02, $p < 2 \times 10^{-16}$), *C9orf72* expansion (HR = 1.40, SE = 0.06, $p = 1.6 \times 10^{-7}$), bulbar onset (HR = 1.62, SE = 0.03, 8.5×10^{-39}), and lower proportion of natural killers (HR = 0.93, SE = 0.02, $p = 7.7 \times 10^{-3}$).

4. Discussion

We here present the most comprehensive analysis of epigenetic age acceleration in amyotrophic lateral sclerosis to date. We estimated biological aging using epigenetic clocks across three generations and calculated the EAA by regressing our model on control individuals to examine case/control status. Additionally, we regressed our model on *C9orf72*-negative ALS patients to assess the impact of *C9orf72* expansion. Our analysis was further supplemented with scores that projected the DunedinPACE pace of aging and telomere length.

Table 2. Overlap between CpG sites used for white blood cell count estimation per sequencing array and the model of epigenetic age acceleration clock.

	Total number of CpGs per *FlowSorted* package	PCGrimAge	Horvath	Pan-mammalian	DunedinPACE	DNAmTL
Total number of CpGs per clock		78464	353	335	173	140
FlowSorted.BloodExtended.EPIC overlap	1200	128	0	0	1	0
FlowSorted.BloodExtended.450k overlap	1500	390	0	1	4	1

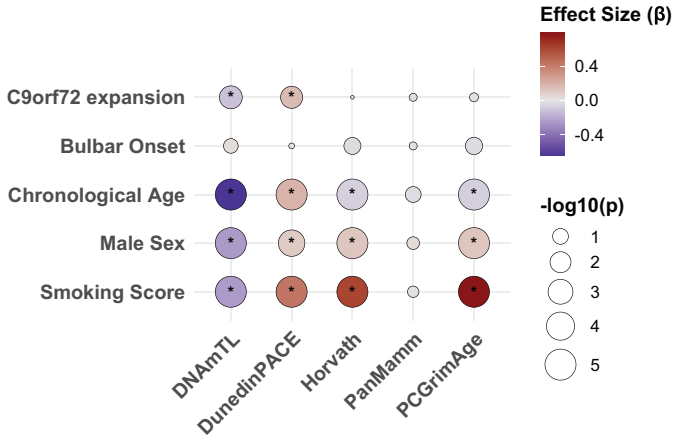

Figure 3. Effect sizes and significance of covariates across models with C9orf72 status adjustment. Each point represents a covariate within a model, with fill color indicating the estimated fixed effect size (β): red for positive, blue for negative, and gray near zero. A positive effect indicates increased biological aging for all EAA parameters, whereas for DNAmTL, the negative effect reflects shorter telomere length, which is also consistent with accelerated aging. Effect sizes are normalized for comparability across all parameters in the model. Point size corresponds to statistical significance, represented as $-\log_{10}$(multiple testing adjusted p) values capped at 5 to facilitate visualization. Asterisks denote covariates with adjusted $p < 0.05$. Covariates include *C9orf72* status, chronological age, sex, batch, site of onset, WBC, and smoking score.

We fitted two models: one that incorporated WBC proportion and another that did not. Our results demonstrated that the model adjusted for WBC proportion fits the data significantly better, indicating that accounting for the immune cell composition improves the accuracy of the associations. When we adjusted for WBC, we found no significant difference between the disease and control groups, except in the case of *C9orf72*-positive ALS patients. We also confirmed a strong link between accelerated aging, male sex, and smoking that has been previously reported in the literature [33,34].

We conclude that the changes in DNA methylation observed in general ALS reflect shifts in WBC composition. These shifts may both confound and contribute to measures of accelerated biological aging. Our study revealed that multiple white blood cell types significantly affect epigenetic age acceleration estimates, with partial overlap between CpGs used to predict PCGrimAge and WBC count. Other studies have also indicated that all generations of epigenetic clocks, including DunedinPACE, are highly sensitive to these changes in WBC proportions, which can lead to spurious inferences on biological age if this is not accounted for [35,36]. At the same time, shifts in WBC composition may themselves be part of the accelerated biological aging process. Aging is linked to a decline in immune system function and changes in WBC composition, such as a decline in the number of naive B and T cells with a shift to the memory cells, a phenomenon known as immunosenescence [37].

Our finding contrasts with those reported previously by Zhao et al. [38]. A significant association between EAA and ALS was reported, as well as for WBC proportions. However, it is important to note that their analyses for both outcomes were performed separately, meaning that the model investigating the relationship with ALS did not adjust for WBC composition. Additionally, their model did not include smoking status, which we also found significantly associated with both ALS risk and epigenetic age acceleration. This may have resulted in residual confounding and stresses the need for standardized analyses in studies on EAA in ALS.

Interestingly, we found evidence that patients with ALS caused by the *C9orf72* repeat expansion experience an increased pace of aging measured with DunedinPACE. This is consistent with a recent MRI study that revealed that ALS patients, particularly those with the *C9orf72* mutation, with cognitive or behavioral impairments, exhibited accelerated brain aging [39]. Additionally, patients with the *C9orf72* repeat expansion have shorter telomere length compared to those without an expansion. This finding is supported by an orthogonal approach that inferred telomere length from whole-genome sequencing, showing that telomeres are shorter in ALS patients with the *C9orf72* repeat expansion [40]. However, we observed no difference in the EAA derived from first-, second-, and third-generation epigenetic clocks among these patients. It is important to note that these clocks differ a lot from the DenedinPACE and DNAmTL by their design, training data, and CpG sites used. For instance, DunedinPACE measures short-term dynamics of aging, while clocks like Horvath and PCGrimAge reflect cumulative, long-term age-related changes. Given the cross-sectional nature of our data, we cannot determine whether the increased pace of aging and shortening of telomeres occurred after the onset of disease or was present before. Thus, the timing and nature of these aging-related changes remain unclear, highlighting the importance of further longitudinal research.

Our study has several potential limitations. First, we used blood-derived methylation data and blood-derived epigenetic clocks to estimate age acceleration related to ALS, which is a neurological disease. While we acknowledge that blood can serve as a proxy for the processes occurring in the brain, more accurate conclusions could be drawn from the brain methylation data. However, unlike other neurological disorders, such as Alzheimer's or Parkinson's disease, we did not observe the signatures of accelerated aging in ALS patients using blood samples. Second, as previously mentioned, our study is cross-sectional and therefore we cannot establish temporal relationships between the changes we observe and ALS disease onset. A future longitudinal study is recommended to clarify these relationships.

Our findings motivate research investigating the role of the white blood cell composition in ALS pathophysiology and changes in this proportion during ALS progression. Previous studies have reported a positive association between ALS and the proportions of granulocytes and monocytes, as well as dysfunction in regulatory T cells [22,41,42]. Together, this evidence suggests that an imbalance in innate immune cells may contribute to both ALS risk and progression. However, the causal relationship of this imbalance needs further investigation. While the concept of accelerated biological aging is still intriguing, future longitudinal studies are required to disentangle its temporal relationship with disease onset and progression.

5. Conclusion

Our study showed no significant difference in the pace of biological aging between ALS patients and controls, except for ALS patients carrying the *C9orf72* mutation. We emphasize the importance of altered white blood cell proportions in general ALS pathophysiology as opposed to accelerated aging per se, but further research is required to explore the causal aspects of this imbalance.

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Author contributions

Polina Merbaum: Conceptualization, formal analysis, investigation, methodology, software, visualization, writing – original draft, writing – review and editing

Ramona Zwamborn: Conceptualization, data curation, methodology, investigation, software, writing – review and editing

Paul Hop: Conceptualization, data curation, methodology, software, writing – review and editing

Project MinE ALS Sequencing Consortium: Data curation, methodology, writing – review and editing

Wouter van Rheenen: Conceptualization, investigation, methodology, resources, supervision, writing – review and editing

Jan Veldink: Conceptualization, methodology, resources, project administration, supervision, funding acquisition, writing – review and editing

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Ethical declaration

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human experimental investigations. In addition, informed consent has been obtained from the participants involved.

Data availability statement

Data obtained in the Netherlands is available at the European Genome-Phenome Archive with an ID EGA00010001938; other data is available upon request through the Project MinE website.

ORCID

Polina Merbaum http://orcid.org/0000-0001-6015-1554
Ramona Zwamborn http://orcid.org/0000-0003-3952-5042
Paul Hop http://orcid.org/0000-0002-9007-7436
Wouter van Rheenen http://orcid.org/0000-0002-5860-1533
Jan Veldink http://orcid.org/0000-0001-5572-9657

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Appendix

Members of Project Mine Consortium

Philip Van Damme[1,2], Philippe Corcia3, Philippe Couratier[4], Patrick Vourc'h[5], Orla Hardiman[6], Russell McLaughin[7], Marc Gotkine[8], Yossef Lerner[8], Yehuda Shovman[8], Vivian Drory[9], Nicola Ticozzi[10,11], Vincenzo Silani[10], Jan H. Veldink[12], Leonard H. van den Berg[12], Mamede de Carvalho[13], Teresa Salas[14], Jesus S. Mora Pardina[15], Monica Povedano[16], Peter Andersen[17], Markus Weber[18], Nazli A. Başak[19], Ammar Al-Chalabi[20], Chris Shaw[20], Pamela J. Shaw[21], Karen E. Morrison[22], John E. Landers[23], Jonathan D. Glass[24], Clifton L. Dalgard[25]

(1) KU Leuven – University of Leuven, Department of Neurosciences, Experimental Neurology, and Leuven Brain Institute (LBI), Leuven, Belgium.
(2) University Hospitals Leuven, Department of Neurology, Leuven, Belgium.
(3) Centre SLA, CHRU de Tours, Tours, France; UMR 1253, iBrain, Université de Tours, Inserm, Tours, France.
(4) Centre SLA CHU Dupuytren Limoges, France.
(5) Service de Biochimie et Biologie moléculaire, CHU de Tours, Tours, France.
(6) Academic Unit of Neurology, Trinity College Dublin, Trinity Biomedical Sciences Institute, Dublin, Republic of Ireland.
(7) Complex Trait Genomics Laboratory, Smurfit Institute of Genetics, Trinity College Dublin, Dublin, Republic of Ireland.
(8) Department of Neurology, Hadassah Medical Organization and Faculty of Medicine, Hebrew University of Jerusalem, Israel.
(9) Department of Neurology, Tel-Aviv Sourasky Medical Centre, Israel.
(10) Department of Neurology, IRCCS Istituto Auxologico Italiano, Milano, Italy.
(11) Department of Pathophysiology and Transplantation, Università degli Studi di Milano, Milan, Italy.
(12) Department of Neurology, UMC Utrecht Brain Center, University Medical Center Utrecht, Utrecht University, Utrecht, The Netherlands.
(13) Instituto de Fisiologia, Instituto de Medicina Molecular,Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal.
(14) ALS Unit, Hospital Universitario La Paz-Carlos III, Madrid, Spain.
(15) ALS Unit, Hospital San Rafael, Madrid, Spain.
(16) la Unitat Funcional de Motoneurona, Cap de Secció de Neurofisiologia, Servei de Neurologia, Hospital Universitario de Bellvitge-IDIBELL.
(17) Department of Clinical Science, Neurosciences, Umeå University, Sweden.
(18) Neuromuscular Diseases Unit/ALS Clinic, Kantonsspital St. Gallen, 9007, St. Gallen, Switzerland.
(19) Koç University, School of Medicine, Molecular Biology and Genetics- KUTTAM, Suna and Inan Kıraç Foundation, Istanbul, Turkey.
(20) Maurice Wohl Clinical Neuroscience Institute, King's College London, Department of Basic and Clinical Neuroscience, London, UK.
(21) Sheffield Institute for Translational Neuroscience (SITraN), University of Sheffield, Sheffield, UK.
(22) School of Medicine, Dentistry and Biomedical Sciences, Queen's University Belfast, UK.
(23) Department of Neurology, University of Massachusetts Medical School, Worcester, MA, USA.
(24) Department of Neurology, Emory University School of Medicine, Atlanta, GA, USA.
(25) The American Genome Center, Uniformed Services University - "America's Medical School", Bethesda, MD, USA