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AMYOTROPHIC LATERAL SCLEROSIS

Genome-wide study of DNA methylation shows alterations in metabolic, inflammatory, and cholesterol pathways in ALS

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Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease with an estimated heritability between 40 and 50%. DNA methylation patterns can serve as proxies of (past) exposures and disease progression, as well as providing a potential mechanism that mediates genetic or environmental risk. Here, we present a blood-based epigenome-wide association study meta-analysis in 9706 samples passing stringent quality control (6763 patients, 2943 controls). We identified a total of 45 differentially methylated positions (DMPs) annotated to 42 genes, which are enriched for pathways and traits related to metabolism, cholesterol biosynthesis, and immunity. We then tested 39 DNA methylation–based proxies of putative ALS risk factors and found that high-density lipoprotein cholesterol, body mass index, white blood cell proportions, and alcohol intake were independently associated with ALS. Integration of these results with our latest genome-wide association study showed that cholesterol biosynthesis was potentially causally related to ALS. Last, DNA methylation at several DMPs and blood cell proportion estimates derived from DNA methylation data were associated with survival rate in patients, suggesting that they might represent indicators of underlying disease processes potentially amenable to therapeutic interventions.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder characterized by progressive degeneration of motor neurons in the brain and spinal cord (*1*). The disease affects about 1 in 350 people, with death typically occurring within 2 to 5 years after onset. The heritability of ALS is estimated to be around 50% (*2*), showing that a considerable portion of the risk could be conferred by environmental and lifestyle risk factors. However, the identification of these factors has proven difficult because of several challenges such as recall and measurement bias, resulting in a large body of literature with conflicting results and only a few established factors related to ALS risk or patient survival (*3*–*6*). Epigenetic patterns, which act at the interface between genes and environment, can serve as proxies of (past) exposures, therefore enabling the study of these exposures and putative risk factors. Moreover, the identification of ALS-associated epigenetic factors could provide insights into disease etiology and disease processes.

DNA methylation is one of the best characterized and most stable epigenetic modifications and plays an important role in gene regulation, genomic stability, and genomic imprinting (*7*–*9*). The development of standardized assays for quantifying DNA methylation has enabled the systematic analysis of associations between methylomic variation and a wide range of human diseases, including cancer, schizophrenia, and various neurodegenerative diseases (*10*, *11*). DNA methylation in whole blood captures a wide range of putative ALS risk factors at a molecular level, including smoking, alcohol intake, body mass index (BMI), biological age, and various metabolic and inflammatory proteins (*12*–*18*). Leveraging DNA methylation as proxies for these risk factors offers several advantages because it is (i) not prone to recall bias (relevant for smoking and alcohol), (ii) may

capture information not (accurately) captured by the self-report (such as passive and past smoking) and provides a quantifiable measure (*19*), and (iii) is relatively stable in the short term [especially relevant for immunological proteins (*18*)]. Moreover, many risk factor studies have been conducted in small samples (*3*, *6*), whereas our large DNA methylation study can provide a well-powered alternative that jointly considers the molecular correlates of many risk factors. We, therefore, performed a blood-based DNA methylation study of ALS incorporating 9706 samples that passed stringent quality control.

RESULTS

Epigenome-wide association study meta-analysis of ALS identifies 45 DMPs

We quantified genome-wide DNA methylation in whole blood from 10,462 individuals using the Illumina HumanMethylation450 (450 k) array (6275 samples) and the Illumina MethylationEPIC (EPIC) array (4187 samples). We merged individual-level DNA methylation array data from 14 countries into four strata (MinE 450 K, MinE EPIC, AUS1, and AUS2; see Materials and Methods and fig. S1). A total of 6763 patients with ALS and 2943 control individuals passed our stringent quality control, which was followed by normalization of signal intensities in each stratum (Table 1, data file S1, and tables S1 to S5). Samples excluded from our analyses did not show different demographic or clinical characteristics compared to the subset selected for analyses (data file S2).

We performed an epigenome-wide association study (EWAS) in each of the four strata using two methods to adjust for known and unknown confounders. First, we used a linear model adjusting for known confounders and a calibrated number of principal components (PCs) to adjust for unknown confounding factors (fig. S2), followed by correction for residual bias and inflation in test statistics using bacon (hereafter referred to as the LB model) (*20*). Second, we used MOA (mixed linear model–based omic association) as implemented in the OSCA software in which the random effect of total genome-wide DNA methylation captures the correlation structure between probes and directly controls for the genomic inflation (*21*). The MOA algorithm did not converge for the AUS2 stratum, resulting in a total sample size of 9459 for the MOA results. Test statistics across strata were combined using an inverse variance-weighted (IVW) fixed-effects meta-analysis (*22*). Inflation of the test statistics was well controlled in both the LB ($\lambda = 1.046$; Fig. 1) and the MOA results, respectively ($\lambda = 0.984$; Fig. 1), and we observed little heterogeneity between strata (figs. S3 to S5). Various sensitivity analyses indicated that the results were robust to changes in analysis strategy, including adjustment for population stratification (10 genetic PCs), using M values instead of β values, using functional normalization (*23*) instead of dasen (*24*), and excluding specific strata or experimental batches (figs. S6 to S8). Last, application of a method that we recently described (*25*) led to the removal of likely cross-hybridizing probes, including four probes that showed high homology to the *C9orf72* repeat locus (fig. S9). In total, 724,712 positions passed quality control

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Table 1. Demographic and clinical characteristics of study population. Shown are numbers (and percentages) of samples that passed quality control.

	Project MinE		External	
	MinE 450 k	MinE EPIC	AUS1*	AUS2*
	(*N* = 4474)	(*N* = 3897)	(*N* = 1088)	(*N* = 247)
Diagnosis				
Control	1436 (32%)	915 (23%)	493 (45%)	99 (40%)
Case	3038 (68%)	2982 (77%)	595 (55%)	148 (60%)
Sex at birth				
Female	1863 (42%)	1700 (44%)	487 (45%)	124 (50%)
Male	2611 (58%)	2197 (56%)	601 (55%)	123 (50%)
Age (years)				
Mean (SD)	63 (± 11)	61 (± 13)	70 (± 12)	
Missing	438 (9.8%)	949 (24.4%)	77 (7.1%)	
Site of onset†				
Bulbar	861 (28%)	739 (25%)	173 (29%)	36 (24%)
Generalized	98 (3%)	112 (4%)	0 (0%)	0 (0%)
Spinal	2023 (67%)	2060 (69%)	0 (0%)	0 (0%)
Thoracic	10 (0%)	5 (0%)	0 (0%)	0 (0%)
Missing	46 (1.5%)	66 (2.2%)	422 (70.9%)	112 (75.7%)
Survival status†				
Alive	437 (14%)	1112 (37%)	516 (87%)	43 (29%)
Dead	2564 (84%)	1845 (62%)	79 (13%)	87 (59%)
Missing	37 (1.2%)	25 (0.8%)	0 (0%)	18 (12.2%)
Survival (months)†‡				
Median (Q1–Q3)	31.4 (31.4–48.9)	31.3 (21.1–47.1)	31.5 (23.6–44.4)	38.3 (25.4–66.5)
Missing	17 (0.7%)	9 (0.5%)	2 (2.5%)	1 (1.1%)
***C9orf72* status**†				
Expanded (≥30)	200 (7%)	155 (5%)		
Normal	2809 (92%)	2780 (93%)		
Missing	29 (1.0%)	47 (1.6%)		

*Data only included in case/control analyses. †Case only. ‡Dead only.

and were included in the meta-analysis. Of these, 332,066 were specific to the EPIC array, and 26,367 were specific to the 450 k array, respectively.

The LB meta-analysis resulted in 44 differentially methylated positions (DMPs) ($P < 9 \times 10^{-8}$; Fig. 1, A and B, Table 2, fig. S10, and data file S3), and the MOA meta-analysis resulted in 11 significant DMPs ($P < 9 \times 10^{-8}$; Fig. 1, C and D, and data file S4) (*26*). The MOA DMPs comprised a subset of the LB DMPs, with the exception of cg01589155, which is annotated to the *C9orf72* locus; this site was significant in MOA ($P = 1.51 \times 10^{-8}$) and just below the significance threshold in the LB results ($P = 2.59 \times 10^{-7}$) (fig. S11). Effect sizes were generally small, and we observed both hypermethylated (51%) and hypomethylated (49%) DMPs associated with ALS (Fig. 1, B and D). On the basis of the nearest gene mapping, these DMPs were annotated to 42 unique genes. In addition, we annotated each site with cis-eQTMs (cis expression quantitative trait methylations) in blood calculated in an external dataset [six Dutch biobanks included in Biobanking and BioMolecular Resources Research Infrastructure (BBMRI) (*27*)]. This revealed that DNA methylation at 18 sites was significantly associated with the expression of at least one nearby gene [false discovery rate (FDR) < 0.05], which included the nearest gene in 14 of 18 sites (Table 2 and data file S5). The DMPs included multiple colocalized positions (<250 kb), including four DMPs in *ZFPM1*, two DMPs in *C9orf72*, two DMPs in *SGSM2*, two DMPs in *TTC38*, two DMPs near *LCK*, and two DMPs in and near *GPR97*. Most of the colocalized DMPs were highly correlated ($|r| > 0.25$), and we also found several distant DMPs to be highly correlated (figs. S12 and S13).

Sensitivity analyses indicate that ALS-associated differential methylation is not driven by genetic variation in cis or trans, riluzole use, or *C9orf72* status

We performed sensitivity analyses to evaluate whether our results were driven by known biological factors associated with ALS or by genetic variation. First, we examined the effects of the *C9orf72* repeat expansion by performing an EWAS meta-analysis excluding

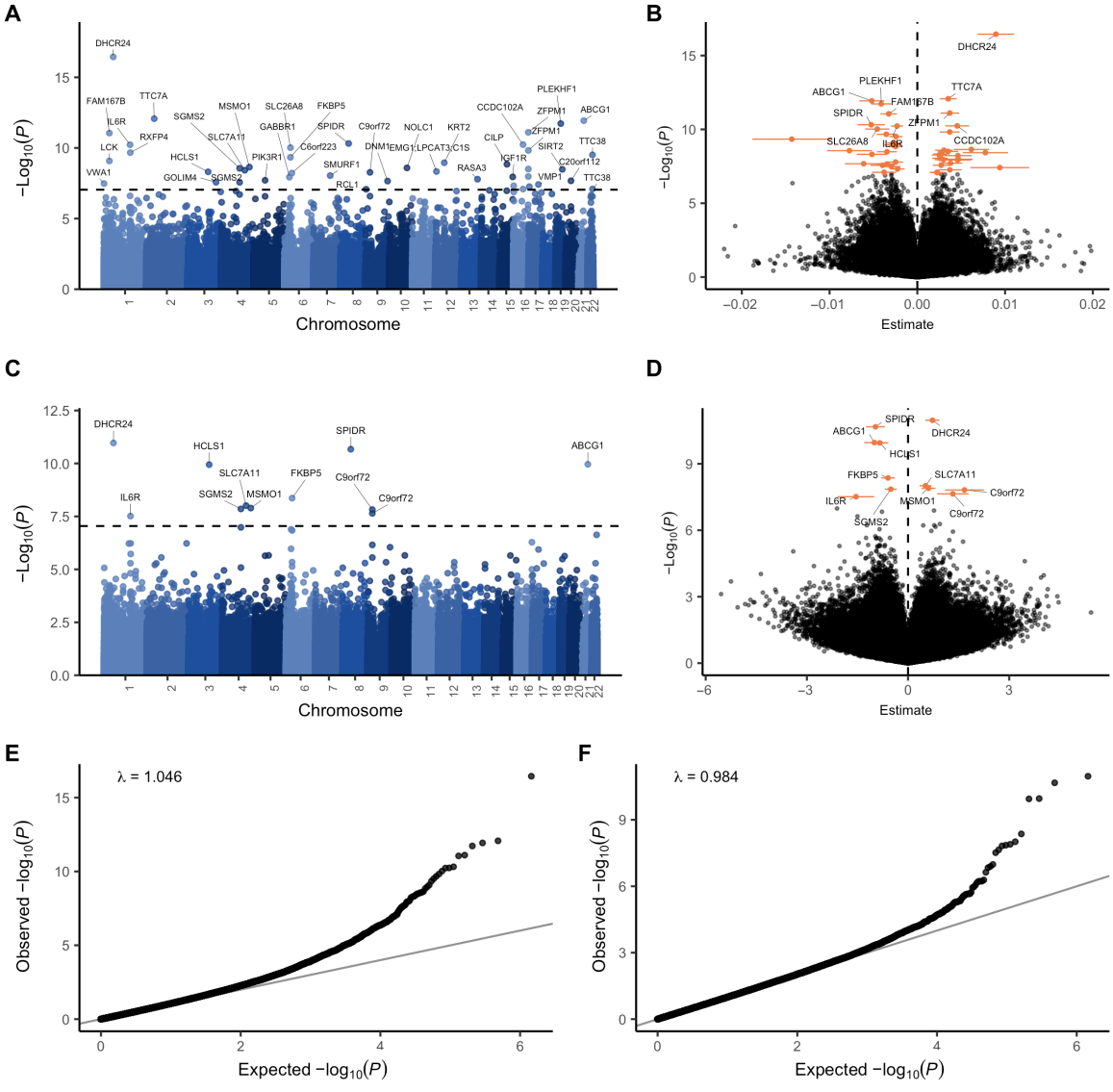

Fig. 1. EWAS meta-analysis. EWAS on 6763 patients and 2943 controls. (**A** and **C**) Manhattan plot comparing (A) LB (linear model + bacon) and (C) OSCA MOA association *P* values [$-\log_{10}(P)$, *y* axis] and genomic location (*x* axis). The dashed line indicates the genome-wide significance threshold (9×10^{-8}). Sites were annotated with the nearest protein-coding gene in ensembl [some gene labels in (A) could not be clearly displayed; all labels are presented in fig. S10]. (**B** and **D**) Volcano plots showing (B) LB and (D) OSCA MOA estimated effect sizes (*x* axis) and association *P* values [$-\log_{10}(P)$, *y* axis]. Ninety-five percent confidence intervals are shown for DMPs, and the nearest genes are shown for the top 10 DMPs identified with the LB algorithm and for all DMPs identified with the MOA algorithm. (**E** and **F**) Quantile-quantile plot showing observed (E) LB and (F) OSCA MOA *P* values [$-\log_{10}(P)$, *y* axis] against the expected distribution under the null (*x* axis).

371 carriers of this mutation. Overall, the results were highly correlated (fig. S14), except for cg01589155 and cg23074747 (located within the *C9orf72* repeat and in a CpG island just upstream of the repeat, respectively), which were strongly driven by *C9orf72* carrier status. Second, to delineate whether DMPs were influenced by riluzole use, we performed an EWAS on riluzole use in patients with ALS (*N* users = 1803, *N* nonusers = 451), finding no evidence of shared signals between the ALS EWAS and the riluzole EWAS (fig. S15). Last, we investigated whether results were driven by genetic variation. For each DMP, we iteratively adjusted for all genetic variants in cis (<250 kb, including variants overlapping the CpG site or probe) as detected in our overlapping whole-genome sequencing (WGS) data (*28*) (N_{ALS} = 5755; $N_{controls}$ = 2184) and blood trans methylation quantitative trait loci (trans-mQTLs)s as reported in the Genetics of DNA Methylation Consortium (GoDMC) database (http://mqtldb.godmc.org.uk). We found no evidence that the DMPs were driven by either genetic variants in cis or in trans (fig. S16).

Enrichment analyses of genes annotated to ALS-associated differential DNA methylation implicate metabolic, inflammatory, and cholesterol pathways

Gene set analysis

To characterize the EWAS results, we performed gene set enrichment analyses based on both nearest genes and cis-eQTMs annotated to each tested position (*29*, *30*). We considered both the default threshold used in the methylGSA package ($P < 0.001$) and the stringent

Table 2. Top 10 DMPs. Details of the 10 most strongly associated sites identified with the LB algorithm. Position, Chromosome:bp (GRCh37); Nearest gene, nearest gene based on GRCh37 (Ensembl release 75); cis-eQTM, the top cis-eQTM for the respective probe; cis-eQTM FDR, *P* value corresponding to the top cis-eQTM, FDR-corrected for the number of tests for the respective probe; *B*, regression coefficient (representing the mean change in β values) and their 95% confidence intervals; *P* value, *P* value from the LB algorithm; PMS indicates that the probe is part of the respective PMS (polymethylation score); Trait, overlap with enriched traits from the MRC-IEU and NGDC EWAS databases (showing a maximum of five traits). HGF, hepatocyte growth factor; N.CDase, neutral ceramidase; FGF.21, fibroblast growth factor 21; CI, confidence interval.

Probe	Position	Nearest gene	cis-eQTM (direction)	cis-eQTM FDR	*B* (95% CI)	*P* value	PMS	Traits
cg17901584	1: 55353706	*DHCR24*	*DHCR24* (–)	2.9×10^{-62}	0.0090 (0.0069–0.011)	3.6×10^{-17}	BMI, HDL-c, and HGF	Hepatic fat, BMI, metabolic trait, and (serum) triglycerides
cg06528816	2: 47242277	*TTC7A*	*TTC7A* (–)	0.13	0.0035 (0.0026–0.0045)	8.5×10^{-13}		Allergic sensitization and total serum IgE
cg06500161	21: 43656587	*ABCG1*	*ABCG1* (–)	1.6×10^{-25}	−0.0052 (−0.0066 to −0.0038)	1.2×10^{-12}	BMI, HDL-c, N.CDase, and FGF.21	Hepatic fat, BMI, metabolic trait, and (serum) triglycerides
cg14945937	19: 30162771	*PLEKHF1*	*PLEKHF1* (–)	0.02	−0.0041 (−0.0053 to −0.003)	1.9×10^{-12}		
cg08940169	16: 88540241	*ZFPM1*	*PIEZO1** (–)	0.08	0.0037 (0.0026–0.0048)	7.8×10^{-12}		Allergic sensitization, total serum IgE, childhood asthma, and schizophrenia
cg07571745	1: 32715428	*FAM167B*	*CCDC28B** (–)	0.26	−0.0033 (−0.0042 to −0.0023)	8.9×10^{-12}		
cg14195992	8: 48265917	*SPIDR*	*SPIDR* (–)	0.0059	−0.0053 (−0.0068 to −0.0037)	4.8×10^{-11}		Birth weight
cg08851837	16: 57558820	*CCDC102A*	*GPR56** (+)	0.84	0.0045 (0.0032–0.0059)	5.8×10^{-11}		
cg09257526	1: 154379696	*IL6R*	*ATP8B2** (–)	0.0031	−0.0023 (−0.003 to −0.0016)	5.9×10^{-11}		Alcohol consumption per day
cg15782984	6: 35993792	*SLC26A8*	*SLC26A8* (–)	0.007	−0.0046 (−0.0059 to −0.0032)	9.5×10^{-11}		

*The association between DNA methylation and the nearest gene was not significant (FDR > 0.05)

genome-wide significance threshold (9×10^{-8}) to select DMPs for enrichment analyses.

We identified two main categories of enriched pathways: First, in both the LB and MOA results, we identified cholesterol/steroid biosynthesis–related pathways. These included the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway steroid biosynthesis and the gene ontology (GO) pathway cholesterol biosynthetic process, sterol biosynthetic process, organic hydroxy compound biosynthetic process, and secondary alcohol biosynthetic process, which were enriched among the MOA results (Table 3). In addition, we found that these and related pathways were enriched among annotated cis-eQTMs in both the LB and MOA results (table S5). The enrichments were mainly driven by four DMPs: three covarying DMPs in *DHCR24* (cg17901584), *MSMO1* (cg05119988), and *ABCG1* (cg06500161) (figs. S12 and S13) and a DMP in *SLC7A11* (cg06690548). Of these, cg17901584, cg05119988, and cg06500161 were strongly associated with the expression of the nearest gene in blood (*DHCR24*, *MSMO1*, and *ABCG1*, respectively; Table 2 and data file S5).

Second, the immune-related KEGG pathways cytokine–cytokine receptor interaction and natural killer (NK) cell–mediated cytotoxicity were enriched in the LB results (at $P < 0.001$) but not in the MOA results (Table 3).

EWAS database enrichments

To further characterize the results, we assessed whether the DMPs overlapped with trait-associated positions reported in publicly available EWAS databases (*31*, *32*). For the LB results, we found a significant overlap (FDR < 0.05) with 23 traits in the MRC Integrative Epidemiology Unit (IEU) database (Fig. 2A and Table 4) and 20 traits in the National Genomics Data Center (NGDC) database (fig. S17 and data files S6 and S7), with a total of 23 of 44 DMPs overlapping with one or more enriched traits. For the MOA results, we found a significant overlap (FDR < 0.05) with 20 traits in the MRC-IEU database

Table 3. Gene set enrichments. Details of the gene sets that were significantly enriched (FDR < 0.05) among the MOA and LB results based on nearest genes annotated to each site. Method, EWAS method and *P* value cutoff applied to the respective EWAS test statistics resulting in the input probes for the shown enrichment analyses; *N* overlap, number of genes that overlap with genes in the respective pathway; *N* genes, total number of genes in the pathway; FDR, FDR-controlled *P* values (Wallenius' noncentral hypergeometric distribution).

Method	Database	Pathway	*N* overlap	*N* genes	FDR
LB ($P < 0.001$)	KEGG	Cytokine-cytokine receptor interaction	36	262	0.0012
	KEGG	NK cell–mediated cytotoxicity	22	108	0.036
MOA ($P < 0.001$)	–	–	–	–	–
LB ($P < 9 \times 10^{-8}$)	–	–	–	–	–
MOA ($P < 9 \times 10^{-8}$)	KEGG	Steroid biosynthesis	2	18	0.015
	GO BP	Cholesterol biosynthetic process	3	71	0.021
	GO BP	Sterol biosynthetic process	3	77	0.021
	GO BP	Organic hydroxy compound biosynthetic process	4	251	0.021
	GO BP	Secondary alcohol biosynthetic process	3	71	0.021

(fig. S18) and 14 traits in the NGDC database (fig. S19), with a total of 8 of 11 DMPs overlapping with one or more of the enriched traits.

Among the strongest enrichments in the MRC-IEU database (all results shown in data files S6 and S7) were BMI, total serum immunoglubulin E (IgE) (only enriched among the LB results), (serum) triglycerides, waist circumference, and high-density lipoprotein cholesterol (HDL-c), of which all showed effect directions opposite to those found for ALS, except for HDL-c (Table 4). Using the Louvain clustering algorithm (*33*), we found that the overlapping traits clustered into two (MOA) to three (LB) clusters. These included two connected cholesterol-related (including HDL-c and triglycerides) and metabolism-related (including BMI and alcohol consumption) clusters, which were identified in the results from both EWAS methods. In addition, in the LB results, we identified an inflammation-related trait cluster that included traits such as total serum IgE and atopy. We found that this inflammation-related cluster was independent of the other clusters, as indicated by iterative analyses presented in Fig. 2B, showing that only the immune-related traits remained significant ($P < 0.05$) after excluding BMI-related probes (figs. S17 to S19).

Polymethylation scores for BMI, HDL-c, alcohol intake, and white blood cell proportions are associated with ALS

To gain further insight into potential intermediate phenotypes associated with ALS, we used 39 published polymethylation scores (PMSs) as proxies for various traits and exposures, including BMI, HDL-c, low-density lipoprotein cholesterol (LDL-c), total cholesterol, alcohol consumption, smoking, white blood cell (WBC) proportions [CD4T, CD8T, monocytes, granulocytes, and NK cells], biological age, and a collection of immunological and neurological proteins (*12*–*18*, *34*, *35*).

First, we performed a validation analysis for each of the PMSs for which we had relevant clinical/exposure data available (see Materials and Methods and table S4). We selected PMSs with an explained variance of ≥5%, as indicated by an incremental R^2 between the null model (including known covariates and control probe PCs) and the model including the respective PMS (Fig. 3A). Two PMSs that were included in the validation analysis did not meet the implemented threshold of ≥5% (LDL-c and total cholesterol).

We found that PMSs for HDL-c, monocyte cell proportion, and granulocyte cell proportion were positively associated with ALS ($P < 1.3 \times 10^{-3}$; Fig. 3, B and C, and data file S8), and the PMSs for alcohol intake, BMI, and the other WBC proportions (CD4T, CD8T, NK, and B cells) were negatively associated with ALS, a result that reflects the nature of proportion data given the positive associations of other cell types ($P < 1.3 \times 10^{-3}$; Fig. 3, B and C, and data file S8). Although we did find a significant association for epigenetic age acceleration [$P = 6.7 \times 10^{-5}$; clock of Zhang *et al.* (*15*) adjusted for chronological age], there was significant heterogeneity between strata (Cochran's Q test $P < 0.1/39$; data file S8), which led us to exclude age acceleration for further consideration. In addition, we considered the multitissue clock of Horvath (*36*) and the clock of Hannum *et al.* (*37*), but the associations for both did not pass the multiple testing threshold ($P = 1.8 \times 10^{-3}$ and $P = 0.23$, respectively, at a multiple testing threshold of 1.3×10^{-3}; fig. S20).

Conditional analyses showed that PMSs HDL-c, BMI, and alcohol were independently associated with ALS, although the HDL-c and BMI associations were attenuated after mutual adjustment (Fig. 3D and fig. S21). The WBC associations also remained significant ($P < 1.3 \times 10^{-3}$) after mutual adjustment for the other PMSs, except for a subset of immunological proteins that attenuated the associations (fig. S22). Adjustment for DMPs showed that signal is shared between several DMPs and ALS-associated PMSs (fig. S23); most notably, the alcohol intake association became not significant ($P > 1.3 \times 10^{-3}$) upon adjustment for two covarying DMPs in *SLC7A11* (cg06690548) and *C6orf223* (cg18120259) (figs. S12 and S13). The HDL-c association became not significant ($P > 1.3 \times 10^{-3}$) upon adjustment for two covarying DMPs in *DHCR24* (cg17901584) and *ABCG1* (cg06500161) (figs. S12 and S13). We assessed whether the associations were primarily driven by carriers of the *C9orf72* repeat expansion but found no evidence that this was the case (fig. S24) nor were the PMS associations primarily driven by specific strata or experimental batches as evidenced by leave-one-out analyses (figs. S25 to S28).

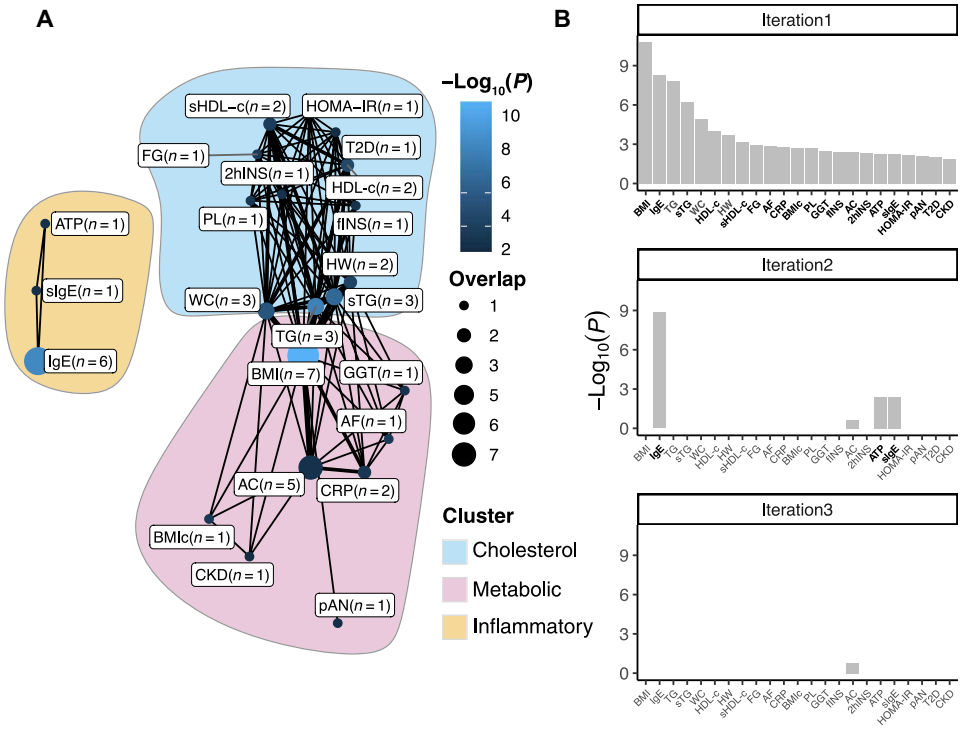

Fig. 2. EWAS database enrichments. Significant overlap (Fisher's exact test, FDR < 0.05) between traits included in the MRC-IEU EWAS database and ALS-associated positions identified using the LB model. (**A**) Network showing the traits that significantly (FDR < 0.05) overlap with the ALS-associated positions. Nodes indicate the overlap between ALS-associated positions and positions associated with indicated traits, with larger nodes indicating more overlap, and lighter shades of blue indicating stronger associations. Edges indicate probe overlap between the traits, with thicker lines indicating more overlapping probes. Colored surfaces indicate the clusters (cholesterol, metabolic, and inflammatory) identified using the Louvain clustering algorithm. (**B**) Identification of independent clusters of traits. The first iteration shows the traits that significantly overlap with the ALS-associated probes at FDR < 0.05. In subsequent iterations, the probes belonging to the trait with the lowest-enrichment *P* value were excluded, and enrichment tests were performed using the remaining traits. This algorithm was repeated, retaining traits that were nominally significant ($P < 0.05$, indicated in bold), until at most one trait remained nominally significant. At the third iteration, no traits remained nominally significant ($P < 0.05$), showing that both BMI and related traits (including triglycerides and HDL-c) and IgE and related traits (atopy) show independent overlap with the ALS-associated positions. IgE, total serum IgE; TG, triglycerides; sTG, serum triglycerides; WC, waist circumference; sHDL-c, serum HDL-c: HW, hypertriglyceridemic waist; FG, fasting glucose; AF, atrial fibrillation; BMIc, BMI change; PL, postprandial lipemia; GGT, gamma-glutamyl transferase; fINS, fasting insulin; AC, alcohol consumption per day; 2hINS, 2-hour insulin; ATP, atopy; sIgE, high serum IgE; pAN, plasma adiponectin; T2D, type 2 diabetes; CKD, chronic kidney disease; HOMA-IR, homeostatic Model Assessment of Insulin Resistance.

Last, in addition to the PC-adjusted models, we also evaluated less stringent models, showing that various immunological and neurological proteins such as C-reactive protein (CRP), interleukin-6 (IL-6), transforming growth factor–α (TGF-α), and chemokine eotaxin-1 (CCL11) as well as smoking were significantly associated with ALS when PCs were excluded ($P < 1.3 \times 10^{-3}$; Fig. 3, E and F, and fig. S29).

Survival analyses indicate that WBC proportions and DNA methylation at five ALS-associated DMPs are associated with disease progression

A total of 5138 patients met the inclusion criteria for the survival analyses (see the "Survival analyses" section in Materials and Methods). Comparison of included and excluded patients (data file S2) shows that both exhibit characteristics that match population-based studies (*38*). This indicates that we have included a representative sample of patients with the entire spectrum of disease characteristics.

We performed multivariate Cox proportional hazards (PHs) meta-analyses on the 45 DMPs identified using the MOA and LB models. A total of five DMPs showed a significant association with survival after correcting for known confounders and PCs ($0.05/45 = P < 1.11 \times 10^{-3}$) and cross-validation between three sensitivity analyses. Effect sizes were moderate and showed both shorter and longer survival time between DNA methylation and overall survival (data file S9).

All reported positions were not affected by the addition of time-varying effects in the Cox PH model or by applying a restricted cubic spline with varying complexity to model the baseline log cumulative hazard (fig. S30). Moreover, after adjusting for *C9orf72* carrier status in the multivariate Cox PH model, the positions (besides the *C9orf72* mapped probe) remained significantly associated with survival ($P < 1.11 \times 10^{-3}$; fig. S30). Four positions showed a significant (FDR < 0.05) cis-eQTM effect with *FKBP5*, *ATP8B2*, *SPIDR*, and *DHCR24* (Table 5).

We also assessed whether the PMSs were associated with survival, finding that a higher proportion of granulocytes was significantly associated with decreased survival and a higher proportion of NK cells was associated with increased survival ($P < 1.3 \times 10^{-3}$; Fig. 3, B and C, bottom; and data file S10). These associations were robust in sensitivity analyses (figs. S31 and S32) and persisted upon adjustment for *C9orf72* carrier status (fig. S33).

DISCUSSION

In this study, we present genome-wide DNA methylation data on more than 10,000 individuals, with extensive clinical data and WGS data available for most of the samples. After thorough quality control and extensive sensitivity analyses, we identified a total of 45 DMPs at which variable DNA methylation is robustly associated with ALS ($P < 9 \times 10^{-8}$). By using enrichment analyses, PMSs, and survival analyses, we highlight a role for metabolic, inflammatory, and cholesterol pathways and identify WBC proportions and several DMPs as potential disease modifiers in ALS.

Genes annotated to DMPs were enriched for pathways related to cholesterol biosynthesis. The main drivers of these enrichments include cg17901584 (*DHCR24*), cg06500161 (*ABCG1*), cg05119988 (*MSMO1*), and cg06690548 (*SLC7A11*), with DNA methylation at the first three positions being associated with expression of their annotated genes in blood. These genes are all involved in cholesterol

Table 4. EWAS database enrichments. Ten strongest enrichments within the MRC-IEU EWAS database. FDR is the FDR-corrected *P* values from a Fisher's exact test. Effect directions indicate whether the ALS EWAS and trait EWAS effect sizes share the same direction of effect (for example, an opposite direction of effect for BMI indicates that DNA methylation changes at overlapping positions associated with a lower BMI are also associated with a higher ALS risk). EWAS method indicates whether DMPs identified with respective method were enriched for the given trait.

Trait	FDR	Effect directions	EWAS method
BMI	1.36×10^{-9}	Opposite	LB and MOA
Total serum IgE	1.93×10^{-7}	Opposite	LB
Triglycerides	4.02×10^{-7}	Opposite	LB and MOA
Serum triglycerides*	1.32×10^{-5}	Opposite	LB and MOA
Waist circumference	1.85×10^{-4}	Opposite	LB and MOA
HDL-c	0.0013	Equal	LB and MOA
Hypertriglyceridemic waist	0.0024	Equal	LB and MOA
Serum HDL-c*	0.0066	Equal	LB and MOA
Fasting glucose	0.0097	Opposite	LB and MOA
Atrial fibrillation	0.011	Opposite	LB and MOA

*Note that we adhered to the trait descriptions as provided in the database: serum, plasma, and whole-blood measurements are included as distinct traits ("triglycerides" and "high-density lipoprotein cholesterol" refer to whole-blood measurements).

biosynthesis and lipid transport, and DNA methylation at these positions has been robustly linked to HDL- and total cholesterol, triglyceride concentration, and BMI-related traits such as diabetes and hepatic fat content (*31*, *32*). Both cg17901584 (*DHCR24*) and cg06500161 (*ABCG1*) are included in the HDL-cholesterol PMS and explain a considerable part of the association that we found between elevated HDL cholesterol and ALS. Moreover, we identified two covarying probes in *SGMS2* (sphingomyelin synthase 2), which is of interest given that altered sphingolipid synthesis has recently been linked to ALS (*39*).

cg06690548 (annotated to *SLC7A11*) has also been previously associated with alcohol intake and related factors such as gamma-glutamyl transferase (GGT) and phosphatidylethanol (*13*, *31*, *32*), and the association between the alcohol PMS and ALS was primarily driven by this DMP. Alcohol has been extensively studied in previous epidemiological studies of risk factors for ALS with varying results, but a recent review suggests that alcohol has a risk-decreasing effect, which is in line with our current results (*40*). Previous work showed that increased DNA methylation at cg06690548 is associated with down-regulation of *SLC7A11* in brain tissue (*41*). *SLC7A11* encodes xCT, a cystine-glutamate antiporter that imports cystine while exporting glutamate, the former being an essential precursor of glutathione, the major antioxidant in the brain. It is possible, therefore, that the association found in *SLC7A11*—and by extension alcohol as risk factor for ALS—is related to two well-established pathologic processes in ALS: glutamate excitotoxicity and/or oxidative stress.

Both the EWAS trait enrichments and PMS analyses indicate that lower BMI is associated with ALS. The BMI association persisted after adjustment for other PMSs, including those for HDL cholesterol and alcohol intake, although these PMSs are not perfect proxies of the respective covariates. Lowered BMI throughout the course of the disease (*42*), as well as various other systemic metabolic alterations, including hypermetabolism and hyperlipidemia (*39*, *43*), have been reported in patients with ALS and mouse models of the disease. Several pathophysiological mechanisms underlying alterations in (lipid) metabolism have been implicated, although it is not clear whether these represent a cause or consequence of the disease. For example, metabolism may be altered because of mitochondrial defects, uncontrolled fasciculations, or increased respiratory effort (*43*). These findings may be connected as patients with ALS may compensate for hypermetabolism by increasing energy intake that could in turn lead to hyperlipidemia (*43*). In addition, the immune alterations that we found may be related to these findings, because it has been shown that metabolism and the immune system are connected (*44*). However, the metabolic- and cholesterol-related findings were statistically independent of the immune-related findings and thus did not support a shared mechanistic pathway. The finding of disrupted metabolic pathways may be a potential avenue for therapeutic intervention, because diet represents a modifiable factor and previous studies in patients with ALS and animals suggest that dietary intervention could benefit disease prognosis, for example, by compensating for defects in lipid metabolism or compensating for increased energy demand or lower BMI (*45*).

It is important to note that our analyses can say little about causality, and we need to be cautious in concluding that these factors represent major risks for the disease. However, Mendelian randomization (MR) analyses in our latest genome-wide association study (GWAS) (*28*) indicate that blood cholesterol is causally related to ALS, whereas no causal evidence for (among others) BMI, triglycerides, blood pressure, and other metabolic traits was found. This shows that the causal role for cholesterol in ALS might be independent of other metabolic traits. Although these MR analyses assessed blood cholesterol, neurons are thought to use similar molecular mechanisms (*46*), and the shared genetic susceptibility between cholesterol and ALS risk could therefore indicate that cholesterol is also raised in the spinal cord and brain. Cholesterol is involved in many crucial processes in the central and peripheral nervous system, including membrane fluidity, synapse formation facilitation, neurite growth, and long-term potentiation (*39*). Alternatively, it has been suggested that the energetic needs of large

A

PMS	Reduced adjusted R^2	Incremental adjusted R^2	Univariate R^2
Age[15,*]	0.13	0.76	0.91
Smoking[16]	0.08	0.32	0.39
Granulocytes[35,*]	0.05	0.77	0.81
Smoking[12,*]	0.08	0.27	0.34
Monocytes[35,*]	0.09	0.45	0.52
Alcohol[13,*]	0.07	0.1	0.07
BMI[14,*]	0	0.08	0.06
BMI[16]	0	0.08	0.08
HDL-c[16,*]	0.13	0.07	0.07
CRP[17,*]	0.05	0.07	0.05
Alcohol[16]	0.07	0.009	0.09
Total Cholesterol[16]	0.08	0.005	0.02
LDL-c[16]	0.07	0	0.01

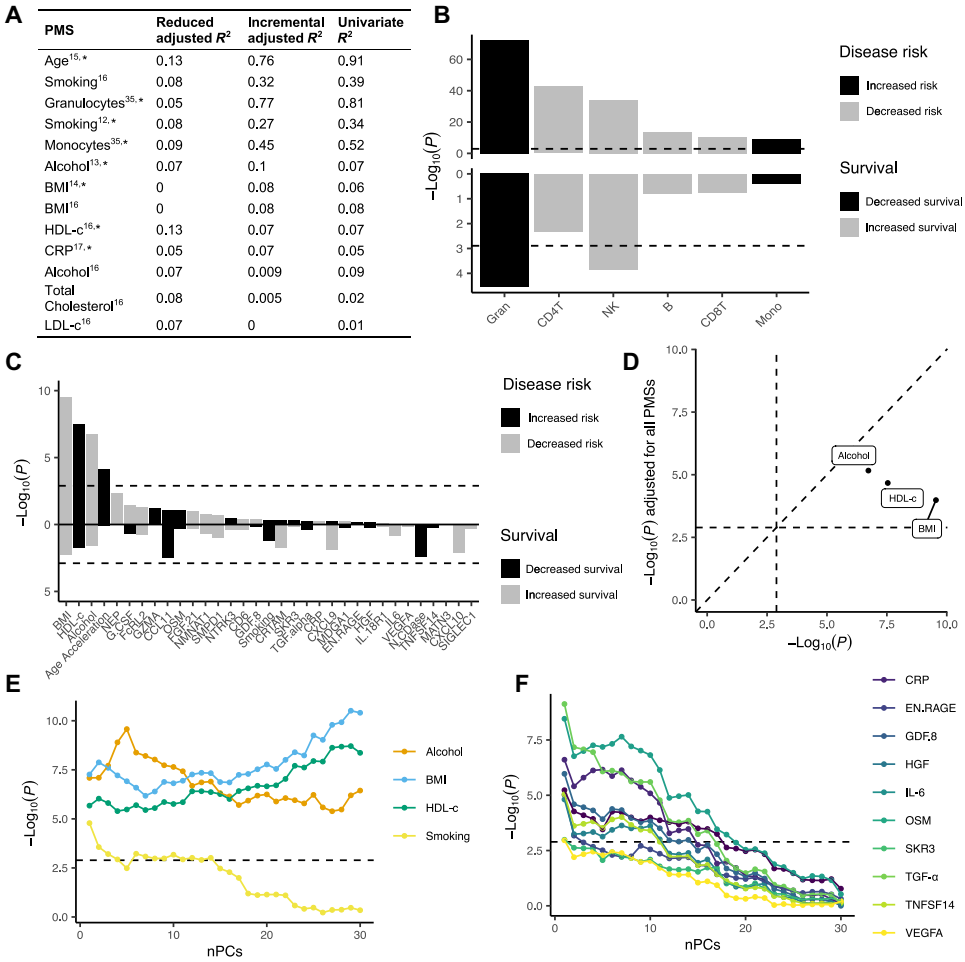

Fig. 3. Polymethylation score analyses on disease risk and patient survival. Polymethylation scores (PMSs) were determined as proxies for various traits, exposures, proteins, and WBC proportions, calculated as weighted sums based on probes and weights derived from published papers, respectively. Case-control association analyses were performed on 6763 patients and 2943 controls; survival analyses were performed within 5162 patients. (**A**) Explained variance of PMSs calculated in samples for which both DNA methylation data and biomarker/clinical data were available ($N = 800$ of 2000). Reduced R^2 represents the variance explained by the null model, whereas the incremental R^2 represents the additional variance explained by the PMS over the null model. Last, the explained variance of the univariate model of the respective PMS is displayed (see Materials and Methods). The asterisk indicates that the PMS was used in the association tests. (**B** and **C**) The top panel shows association *P* values from logistic regression [$-\log_{10}(P)$, *y* axis] for each PMS (*x* axis). (B) WBC proportions and (**C**) various traits and exposures, colored by whether a higher score is associated with increased (black) or decreased (gray) disease risk. The bottom panel shows the Cox PH *P* values [$-\log_{10}(P)$, *y* axis] for each PMS (*x* axis), colored by whether a higher score is associated with decreased (black) or increased (gray) survival, respectively. The dashed line indicates the significance threshold (1.3×10^{-3}). (**D**) Original *P* values [$-\log_{10}(P)$, *x* axis] compared to *P* values after including all PMSs as fixed covariates in the logistic regression model [$-\log_{10}(P)$, *y* axis] for the ALS-associated traits/exposures. (**E** and **F**) Association *P* values [$-\log_{10}(P)$, *y* axis] upon incrementally adding principal components (PCs) as fixed covariates in the logistic regression model. HGF, hepatocyte growth factor; EN.RAGE, extracellular newly identified RAGE-binding protein; GDF8, growth/differentiation factor 8; OSM, Oncostatin-M, SKR3, Serine/threonine-protein kinase receptor R3; TNFSF14, tumor necrosis factor ligand superfamily member 14; VEGFA, vascular endothelial growth factor A; nPCs, number of principal components.

Our results also point toward a role for the immune system in ALS. The EWAS results were enriched for immune-related traits including IgE and allergic sensitization; these results were independent of predicted WBC proportions. DMPs driving these enrichments included, among others, cg06528816 (annotated to *TTC7A*) and a cluster of three covarying DMPs in the *ZFPM1* gene, both implicated in immune-related traits such as IgE, asthma, and allergic sensitization (*31*, *32*). Our PMS analyses corroborate the role of immunity in ALS because we found that WBC proportions were altered in ALS, with a higher ratio of granulocytes and a lower ratio of lymphocytes in patients with ALS (CD4T, CD8T, and NK cells). We further found that increased granulocyte proportions are associated with worse prognosis, whereas NK cell proportions are associated with better prognosis, indicating that WBC proportions might have prognostic value. The role of immunity is further supported by our observation that various PMSs for various inflammatory proteins including CRP, IL-6, TGF-α, and CCL11 were elevated in patients with ALS; although these differences remained after adjustment for WBC proportions, they disappeared upon adjustment for principal components. Our findings are in line with previous studies that identified higher ratios of neutrophils and/or granulocytes to lymphocytes in patients with ALS, elevated inflammatory proteins, and an association between higher neutrophil proportions and worse prognosis (*50*, *51*). Although immune alterations could be part of a systemic aspect of ALS, there is evidence that suggests that the peripheral immune system contributes to neuroinflammation, the latter being an established phenomenon in ALS as well as other neurodegenerative diseases (*50*). Especially interesting in this regard are recent analyses showing that mast cells infiltrate skeletal muscles at the neuromuscular junction and degranulate to help recruit neutrophils (*50*), which prevent reinnervation capacity and may thus be a potential mechanism causing worse prognosis. In line with this, we identified an enrichment for IgE (and related traits such as allergy and atopy), which activate mast cells, and found that increased proportions of granulocytes were associated with ALS and patient survival. Thus, these findings could be of interest for new treatments, especially given that mast cell activity can be influenced therapeutically (*50*).

motor neurons make it selectively vulnerable for alterations in metabolism or could be the source of oxidative stress (*47*). Moreover, lipid concentration in the blood and autophagy are related (*48*), as illustrated by a recent study showing that high cholesterol leads to increased protein aggregation through autophagy impairment in mouse models of Alzheimer's disease (*49*).

Table 5. DMPs associated with survival. Details of the positions significantly associated with survival ($P < 1.11 \times 10^{-3}$). Position, Chromosome:bp (GRCh37); Nearest gene, nearest gene based on Ensembl GRCh37 (75); cis-eQTM, the top cis-eQTM for the respective probe; cis-eQTM FDR, *P* value corresponding to the top cis-eQTM, FDR-corrected for the number of tests for the respective probe; PMS, probe is part of the respective PMS (polymethylation score); HR, hazard ratio; Trait, overlap with significantly enriched traits (FDR < 0.05) from the MRC-IEU and NGDC EWAS databases (showing a maximum of five traits). HGF, hepatocyte growth factor. Survival analyses were performed within 5162 patients.

Probe	Position	Nearest gene	cis-eQTM (direction)	cis-eQTM FDR	HR (95% CI)	*P* value	PMS	Traits
cg14195992	8:48265917	*SPIDR*	*SPIDR* (–)	0.0059	0.07 (0.025–0.2)	4.7×10^{-7}		
cg03546163	6:35654363	*FKBP5*	*FKBP5* (–)	0.016	0.19 (0.087–0.41)	2.7×10^{-5}	HDL-c	BMI, waist circumference, alcohol consumption per day, and chronic kidney disease
cg09257526	1:154379696	*IL6R*	*ATP8B2** (–)	0.0031	0.0049 (0.00045–0.053)	1.3×10^{-5}		Alcohol consumption per day
cg17901584	1:55353706	*DHCR24*	*DHCR24* (–)	2.9×10^{-62}	4.6 (2.2–9.8)	1.0×10^{-5}	BMI, HDL-c, HGF	Hepatic fat, BMI, metabolic trait, and (serum) triglycerides
cg01589155	9:27573532	*C9orf72*			47 (6.2–360)	2.0×10^{-4}		

*The association between DNA methylation and the nearest gene was not significant (FDR > 0.05).

We do not replicate the recently reported association between epigenetic age acceleration and survival (*52*). In our analyses, we adjusted for sampling age, because it has been shown to be crucial when studying epigenetic age acceleration (*53*), especially given that age of onset affects disease progression in ALS (*1*). As we have shown, both survival and age of onset were associated with age acceleration when sampling age was not accounted for, but the associations disappeared upon adjustment. In addition, in our case/control analysis, we observed substantial heterogeneity among strata; hence, our results do not support an unambiguous role for age acceleration in ALS.

We must acknowledge the limitations of our study. First, our cross-sectional design hinders inferences about causality. MR analyses presented in our recent GWAS (*28*) did not find evidence for a causal role of the DMPs identified in this study; although this may indicate a lack of power, it could also indicate that the results reflect the consequences of disease processes rather than causal mechanisms. In that case, the value of the identified DNA methylation changes would lie primarily in revealing underlying disease processes in ALS. Furthermore, the identified ALS- and survival-associated DNA methylation patterns could be of interest as potential starting points for new disease-modifying treatments.

Second, we note that we collected DNA from whole blood rather than from brain tissue. Although some blood DNA methylation patterns reflect those in brain tissue more closely than others—as previously shown for the DMPs that we identified in the *C9orf72* locus (*54*)—DNA methylation is often tissue specific (*55*). However, in contrast to brain tissue, blood DNA methylation is accessible, allowing for sampling close to disease onset and in large numbers. Leveraging the large body of literature available on blood DNA methylation allowed us to uncover risk factors and pathways related to ALS.

Last, the stringent adjustment for confounding that we applied by using PCs and random effects models [OSCA (*21*)] may have obscured biological signals of interest. For example, our results indicate that the additional DMPs identified using the LB algorithm are enriched for inflammatory pathways and traits, which corroborates previous findings that suggest that uncaptured variation can be explained by cell type heterogeneity and related immune processes (*56*). Similarly, we show that the associations found for various immunological proteins such as CRP and IL-6 disappeared upon PC adjustment. This relates to the discussion on whether to treat variables such as cell type proportions as nuisance variables in an EWAS or view them as variables that provide valuable information in themselves (*57*). In this study, we therefore struck a balance by opting for a two-way approach, combining a stringently corrected EWAS with a more targeted approach where we studied "confounders" such as WBC proportions, smoking, and BMI as outcomes of interest, assessing them with both stringent (including PCs) and more lenient models.

MATERIALS AND METHODS

Study design

This study aimed to identify differential DNA methylation in patients diagnosed with definite, probable, and probable laboratory-supported ALS according to the revised El Escorial Criteria (*58*). First, we implemented a comprehensive pipeline tailored to large-scale epigenome-wide studies to identify individually methylated positions in 6763 patients with ALS and 2943 controls without motor neuron

diseases. We explored the biological meaning of the results by performing gene set enrichment analyses and by overlapping our results with trait-associated positions reported in publicly available EWAS databases. Power analysis calculated with the EPIC array online tool (*26*) showed that for 96.6% of sites, we had >80% power to detect a mean DNA methylation difference of 1% using the default significance threshold ($P < 9 \times 10^{-8}$). Second, we applied 39 DNA methylation–based proxies of putative ALS risk factors. Last, we leveraged clinical data to perform survival analysis and reveal indicators of disease progression.

Samples were collected across 14 countries (2:1 case/control ratio). Population-based controls were matched for age, sex, and geographical region in a 1:2 ratio and not screened for (subclinical) signs of ALS. Experimental batches were processed in the same laboratory and sequenced in the same series depicting the origin of each DNA sample, resulting in 44 independent batches after quality control. Strata, for analyses, were defined as samples within the Project MinE sequencing consortium stratified by array technology (MinE 450 k and MinE EPIC), and the external Australian data were stratified into two strata based on differences in signal intensities (AUS1 and AUS2) (see Supplementary Materials and Methods "QC and normalization" section, fig. S1, and QC figure 50 for more details).

DNA methylation was quantified using Illumina 450 k and EPIC arrays. We applied extensive quality control leading to the exclusion of 756 (7.2%) samples (based on several technical metrics, relatedness, genotype concordance, and sex concordance) and 175,134 (24%) probes (based on technical metrics, cross-reactivity, and overlap with common single-nucleotide polymorphisms). For further details on cohorts and QC, see Supplementary Materials and Methods. The investigators were not blinded to the experimental conditions during experiments and the analyses.

Statistical analysis

Epigenome-wide association study

Two approaches were used to perform EWAS analyses:

1)Linear regression was performed at each site, testing for an association between DNA methylation β values and case-control status, adjusting for the following fixed covariates: sex, experimental batch, predicted age, estimated WBC, 30 control probe PCs, and *m* array-wide residual PCs (see Supplementary Materials and Methods). The number of array-wide PCs (*m*) was optimized in each stratum by evaluating the sample-size normalized inflation factors (λ_{1000}). The number of PCs (*m*) were chosen so that for each stratum, $\lambda_{1000} \leq 1.15$ (*m* is 30, 15, 25, and 30 for the MinE 450 k, MinE EPIC, AUS1, and AUS2 strata, respectively). We then corrected for remaining inflation and/or bias in test statistics of each stratum using the bacon algorithm (*20*). Hereafter, we refer to this model as the LB model.

2)Mixed linear model analyses were performed using the MOA algorithm implemented in the OSCA software (v0.45) (*21*). This method tests for an association between case-control status and DNA methylation at a given position, adjusting for both fixed effects (we included predicted age, sex, and experimental batch) and a random genome-wide DNA methylation factor per person with variance-covariance matrix between individuals built from genome-wide DNA methylation sites (*11*, *21*, *59*).

For both the linear model and MOA results, test statistics across strata were combined using an IVW fixed-effects meta-analysis (*22*). Positions with a two-tailed *P* value of $<9 \times 10^{-8}$ were considered genome-wide significant and termed DMPs (*26*). DMPs were considered significantly heterogeneous when Cochran's Q *P* values < 0.1 (corrected for the number of DMPs).

Cis-eQTM analyses

For each position, we tested for an association between DNA methylation and gene expression of genes in cis (<250 kb) using linear regression, adjusting for age, sex, strata, WBC composition, and 20 PCs as fixed effects (10 PCs derived from gene expression data and 10 PCs derived from the DNA methylation data) (*27*). We corrected the test statistics for bias and inflation (estimated on the basis of the association between DNA methylation and expression of all genes using the bacon algorithm). For each site, two-tailed *P* values were corrected for the number of tested genes using FDR correction.

Correlation analyses

β values were first adjusted for the covariates used in the LB algorithm; pairwise correlations were calculated among the residuals of this regression using Pearson's correlation coefficient. Correlations were calculated per stratum (within ALS cases) and combined in an IVW meta-analysis of Fisher's *z*-transformed correlation values (*22*).

Enrichment analyses

Gene set analyses were performed using the Wallenius' noncentral hypergeometric distribution (*29*, *30*). This method takes into account that the number of CpGs assigned to each gene differs by accounting for the probability of a gene being selected using Wallenius' noncentral hypergeometric distribution. Two-tailed Fisher's exact tests were used for trait enrichment analyses. Resulting *P* values from the enrichment analyses were corrected for multiple testing using FDR correction. The filtering procedure for gene sets and traits and the backgrounds used are described in Supplementary Materials and Methods.

PMS analyses

Incremental R^2 estimates from linear regression were used to determine whether the PMS increased the predictive ability above and beyond that of the null model that included the phenotype measure as the dependent variable and case-control status, predicted age, sex, experimental batch, WBC, and 30 control probe PCs as independent variables. For each stratum, we tested for an association between the PMS and case/control status using logistic regression. Sex, predicted age, WBC, experimental batch, 30 control probe PCs, and *m* array-wide PCs (see the "Epigenome-wide association study" section) were included as fixed covariates for all PMSs except for DNA methylation age and WBC proportions. For DNA methylation age, we additionally adjusted for chronological age (representing age acceleration). For the WBC PMSs, we did not adjust for array-wide PCs because these essentially represent WBC proportions (*56*). Strata test statistics were combined using an IVW fixed-effects meta-analysis (*22*). We corrected for the number of PMSs tested using the Bonferroni correction [two-tailed *P* value of $<1.3 \times 10^{-3}$ (0.05/39)]. PMSs were considered significantly heterogeneous when Cochran's Q *P* values $< 2.3 \times 10^{-3}$ (0.01/39).

Survival analyses

We used a multivariate Cox PH regression model to test for an association between survival and DMPs and PMSs, adjusting for predicted age, sex, experimental batch, WBC, 30 control probe PCs, and *m* array-wide PCs (see the "Epigenome-wide association study" section). The PH assumption of the Cox model was checked using Schoenfeld and martingale residuals. In addition, the Royston-Parmar spline model was performed using the flexsurvspline function from the R package flexsurv. Model complexity was assessed by the addition of up to five knots compared to one single knot. Test statistics were combined using IVW fixed-effects meta-analysis.

We corrected for the number of tests using the Bonferroni correction (two-tailed $P < 1.11 \times 10^{-3}$ for DMPs and two-tailed $P < 1.3 \times 10^{-3}$ for PMSs). Positions were considered significantly heterogeneous when Cochran's Q P values < 0.1 (corrected for the number of tests performed).

SUPPLEMENTARY MATERIALS

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Materials and Methods
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View/request a protocol for this paper from *Bio-protocol*.

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Genome-wide study of DNA methylation shows alterations in metabolic, inflammatory, and cholesterol pathways in ALS

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Understanding ALS epigenetics

Neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS), have been associated to epigenetic modifications, suggesting that identification of specific epigenetic patterns could provide insights into disease pathophysiology and help the identification of potential pharmacological targets. Here, Hop *et al.* analyzed DNA methylation pattern, one of the most characterized epigenetic modifications, in almost 10,000 individuals with ALS and controls and identified 45 differentially methylated positions (DMPs) annotated to 42 genes. The changes involved genes associated to metabolic and inflammatory pathways, and the authors identified several DMPs associated with disease progression in their cohort. The results will help the identification of disease-relevant mechanisms that could be targeted to block or delay ALS progression.