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

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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 450k	**MinE EPIC**	**AUS_batch1 ****	**AUS_batch2 ****
	(N=4,474)	**(N=3,897)**	**(N=1,088)**	**(N=247)**
Diagnosis				
Control	1,436 (32 %)	915 (23 %)	493 (45 %)	99 (40 %)
Case	3,038 (68 %)	2,982 (77 %)	595 (55 %)	148 (60 %)
Sex at birth				
Female	1,863 (42 %)	1,700 (44 %)	487 (45 %)	124 (50 %)
Male	2,611 (58 %)	2,197 (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 %)

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Generalized	98 (3 %)	112 (4 %)	0 (0 %)	0 (0 %)
Spinal	2,023 (67 %)	2,060 (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 %)	1,112 (37 %)	516 (87 %)	43 (29 %)
Dead	2,564 (84 %)	1,845 (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	2,809 (92 %)	2,780 (93 %)		
Missing	29 (1.0 %)	47 (1.6 %)		

*** data only included in case/control analyses*

** case only*

† dead-only

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Figure 1 - EWAS meta-analysis: Epigenome-wide association study on 6,763 patients and 2,943 controls. **(a,b)** Manhattan plot comparing **(a)** LB (linear model + *bacon*) and **(b)** 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. **(c,d)** QQ-plot showing observed **(c)** LB and **(d)** OSCA MOA *P*-values ($-\log_{10}(P)$, y-axis) against the expected distribution under the null (x-axis).

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Table 2 - Top ten significant sites:

Details of the ten most significant sites identified with the LB algorithm. Position = Chromosome:bp (GRCh37), Nearest gene = nearest gene based on Ensembl GRCh37 (75), eQTM = the most significant eQTM for the respective probe, eQTM *FDR* = *p*-value corresponding to the most significant eQTM, FDR-corrected for the number of tests for the respective probe, PMS = Indicates that the probe is part of the respective PMS (poly-methylation score), Trait = Overlap with significantly enriched traits from the MRC-IEU and NGDC EWAS databases (showing a maximum of five traits). Abbreviations: HGF = Hepatocyte growth factor, N.CDase = Neutral ceramidase, FGF.21 = Fibroblast growth factor 21.

Probe	Position	Nearest gene	eQTM (direction)	eQTM *FDR*	b	se	P-value	PMS	Traits
cg17901584	1: 55353706	*DHCR24*	*DHCR24* (-)	2.9×10^{-62}	0.0090	0.00110	3.6×10^{-17}	BMI, HDLchol, HGF	Hepatic fat, BMI, Metabolic trait, (serum) Triglycerides
cg06528816	2: 47242277	*TTC7A*	*TTC7A* (-)	0.13	0.0035	0.00049	8.5×10^{-13}		Allergic sensitization, Total serum IgE
cg06500161	21: 43656587	*ABCG1*	*ABCG1* (-)	1.6×10^{-25}	-0.0052	0.00073	1.2×10^{-12}	BMI, HDLchol, N.CDase, FGF.21	Hepatic fat, BMI, Metabolic trait, (serum) Triglycerides
cg14945937	19: 30162771	*PLEKHF1*	*PLEKHF1* (-)	0.02	-0.0041	0.00058	1.9×10^{-12}		
cg08940169	16: 88540241	*ZFPM1*	*PIEZO1*† (-)	0.08	0.0037	0.00054	7.8×10^{-12}		Allergic sensitization, Total serum IgE, Childhood asthma, Schizophrenia
cg07571745	1: 32715428	*FAM167B*	*CCDC28B*† (-)	0.26	-0.0033	0.00048	8.9×10^{-12}		
cg14195992	8: 48265917	*SPIDR*	*SPIDR* (-)	0.0059	-0.0053	0.00080	4.8×10^{-11}		Birth weight
cg08851837	16: 57558820	*CCDC102A*	*GPR56*† (+)	0.84	0.0045	0.00069	5.8×10^{-11}		
cg09257526	1: 154379696	*IL6R*	*ATP8B2*† (-)	0.0031	-0.0023	0.00035	5.9×10^{-11}		Alcohol consumption per day
cg15782984	6: 35993792	*SLC26A8*	*SLC26A8* (-)	0.007	-0.0046	0.00070	9.5×10^{-11}		

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

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Table 3 - Gene set enrichments: Details of the gene sets that were significantly enriched 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 significant genes that overlap with genes in the respective pathway, N genes = Total number of genes in the pathway, *FDR* = FDR-controlled (False discovery rate) *P*-values.

Method	Database	Pathway	N overlap	N genes	*FDR*
LB ($p < 0.001$)	KEGG	Cytokine-cytokine receptor interaction	36	262	0.0012
	KEGG	Natural killer 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

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Table 4 - EWAS database enrichments: Ten most significant trait enrichments within the MRC-IEU EWAS database. *FDR* = FDR-corrected *P*-values (False discovery rate). Effect directions = indicate whether the ALS EWAS and trait EWAS effect sizes share the same direction of effect (e.g. an opposite direction of effect for Body mass index indicates that DNA methylation changes at overlapping sites associated with a *lower* BMI are also associated with a *higher* ALS risk); EWAS method = indicates whether significant sites identified with respective method were enriched for the given trait.

Trait	***FDR***	**Effect directions**	**EWAS method**
Body mass index	1.36×10^{-9}	opposite	LB & MOA
Total serum IgE	1.93×10^{-7}	opposite	LB
Triglycerides	4.02×10^{-7}	opposite	LB & MOA
Serum triglycerides*	1.32×10^{-5}	opposite	LB & MOA
Waist circumference	1.85×10^{-4}	opposite	LB & MOA
High-density lipoprotein cholesterol	0.0013	equal	LB & MOA
Hypertriglyceridemic waist	0.0024	equal	LB & MOA
Serum high-density lipoprotein cholesterol*	0.0066	equal	LB & MOA
Fasting glucose	0.0097	opposite	LB & MOA
Atrial fibrillation	0.011	opposite	LB & 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).

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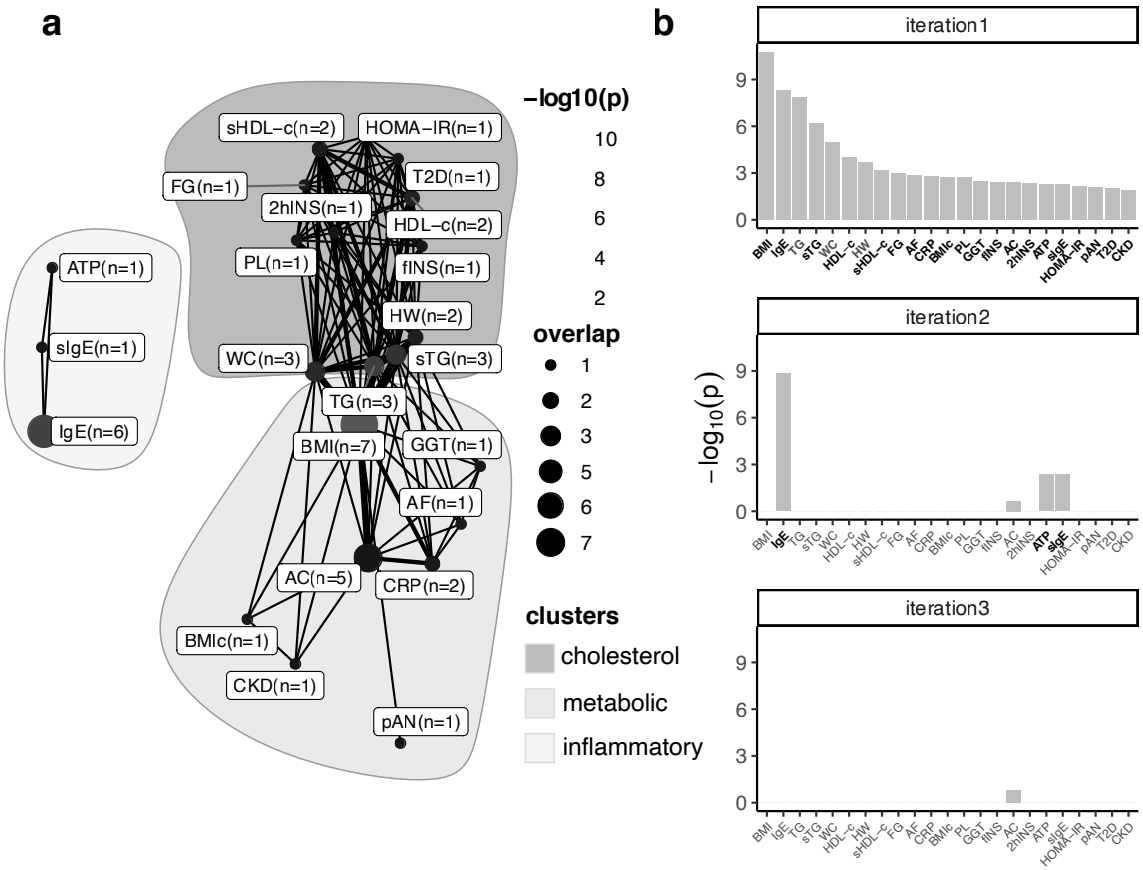

Figure 2 - EWAS database enrichments: Significant overlap between traits included in the MRC-IEU EWAS database and ALS-associated sites identified using the LB model **(a)** Network showing the traits that significantly overlap with the ALS-associated sites. Nodes indicate the overlap between ALS-associated sites and sites 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 most significant trait were excluded and enrichments 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 significant. At the third iteration no traits remained significant, 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 sites.

Abbreviations: IgE = total serum IgE, TG = triglycerides, sTG= serum triglycerides, WC = waist circumference, sHDL-c = serum HDL-c, HW = Hypertriglyceridemic waist, FG = fasting glucose, AF =

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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 II diabetes, CKD = Chronic kidney disease, HOMA-IR = Homeostatic Model Assessment of Insulin Resistance.

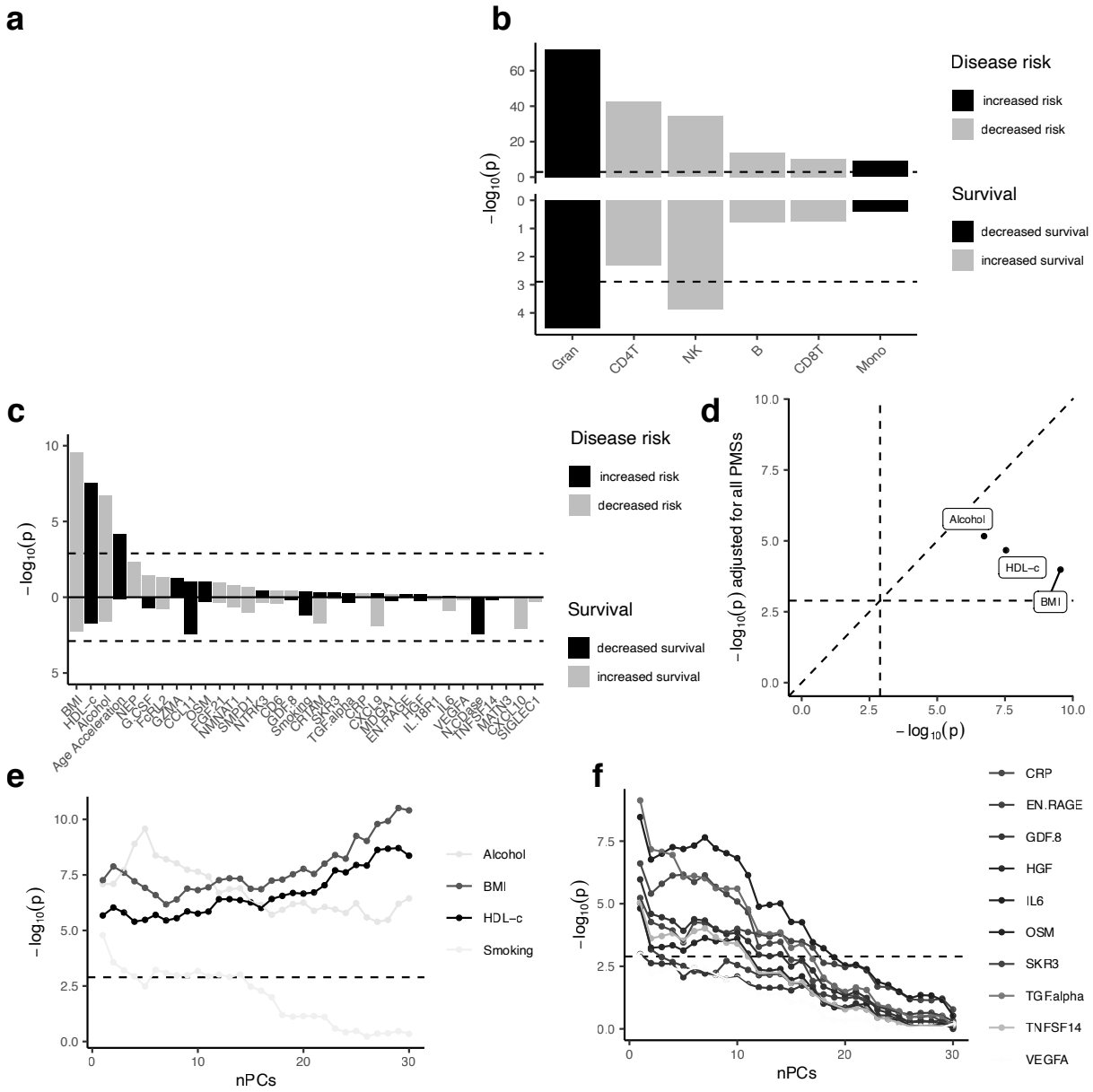

Figure 3 - Poly-methylation score analyses on disease risk and patient survival:

Poly-methylation scores (PMS) were determined as proxies for various traits, exposures, proteins and white blood cell proportions, calculated as weighted sums based on probes and weights derived from published papers respectively.

(a) Explained variance of PMSs calculated in samples for which both DNA methylation data and biomarker/clinical data were available (N=800/2000). Reduced R^2 represents the variance explained by

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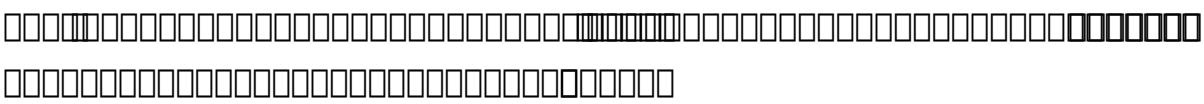
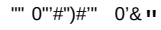
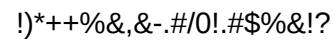
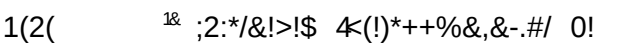
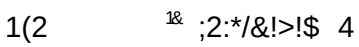
the null model while the incremental R^2 represents the additional variance explained by the PMS over the null model. Lastly, the explained variance of the univariate model of the respective PMS is displayed (see Methods). The asterisk indicates that the PMS was used in the association tests.
(b,c) The upper panel shows association *P*-values ($-\log_{10}(P)$, y-axis) for each PMS (x-axis), **(b)** white blood cell proportions and **(c)** various traits and exposures, colored by whether a higher score is associated with increased (black) or decreased (grey) disease risk. The lower panel shows the Cox proportional hazard *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 (grey) 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 in the logistic regression model ($-\log_{10}(P)$, y-axis) for the significant traits/exposures.
(e, f) Associations *P*-values ($-\log_{10}(P)$, y-axis) upon incrementally adding principal components (PCs) to the logistic regression model. !











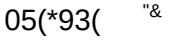


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Table 5 - Significant sites associated with survival: Details of the sites significantly associated with survival. Position = Chromosome:bp (GRCh37), Nearest gene = nearest gene based on Ensembl GRCh37 (75), eQTM = the most significant eQTM for the respective probe, eQTM *FDR* = *p*-value corresponding to the most significant eQTM, FDR-corrected for the number of tests for the respective probe, PMS = Probe is part of the respective PMS (poly-methylation score), HR = Hazard Ratio, Trait = Overlap with significantly enriched traits from the MRC-IEU and NGDC EWAS databases (showing a maximum of five traits). Abbreviations: HGF = Hepatocyte growth factor.

Probe	Position	Nearest gene	eQTM (direction)	eQTM *FDR*	HR	P-value	PMS	Traits
cg14195992	8:48265917	*SPIDR*	*SPIDR* (-)	0.0059	0.074	4.7×10^{-7}		
cg03546163	6:35654363	*FKBP5*	*FKBP5* (-)	0.016	0.19	2.7×10^{-5}	HDLchol	Body mass index, Waist circumference, Alcohol consumption per day, Chronic kidney disease
cg09257526	1:154379696	*IL6R*	*ATP8B2*† (-)	0.0031	0.0048	1.3×10^{-5}		Alcohol consumption per day
cg17901584	1:55353706	*DHCR24*	*DHCR24* (-)	2.9×10^{-62}	4.62	1.0×10^{-5}	BMI, HDLchol, HGF	Hepatic fat, BMI, Metabolic trait, (serum) Triglycerides
cg01589155	9:27573532	*C9orf72*			46.99	2.0×10^{-4}		

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

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Regardless of the exact etiologic role

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$$\lambda_{1000} = 1 + (\lambda_{obs} - 1) * (\frac{1}{n_{cases}} + \frac{1}{n_{controls}}) / (\frac{1}{n_{cases,1000}} + \frac{1}{n_{controls,1000}})$$

$$DNAm_i = Phenotype + Predicted\,Age + Sex + Batch + WBC_{est} + PC1-30_{ctrl-probes} + PC1-m$$

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$$DNAm_i = Case/control\ status + Predicted\ Age + Sex + Batch + WBC_{est} + PC1 - 30_{ctrl-probes} - m + + PC1 - m + SNP_j$$

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$$PMS = \sum_{i}^{m} \beta_i \times CpG_i$$

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$$Phenotype = PMS + Predicted\,Age + Sex + Batch + WBC_{est} + PC1 - 30_{ctrl-probes}$$

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$$Case/control\ status = PMS + Predicted\ Age + Sex + Batch + WBC_{est} + PC1 - 30_{ctrl-probes} + PC1 - m$$

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$$Case/control\ status = Predicted\ Age + Age\ at\ blood\ draw + Sex + Batch + WBC_{est} + PC1 - 30_{ctrl-probes} + PC1 - m$$

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$$Case/control\ status = WBC + Predicted\ Age + Sex + Batch + WBC_{est} + PC1 - 30_{ctrl-probes}$$

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Data availability

Acknowledgments

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Author Contributions

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Competing Interests

personal fees from Cytokinetics, outside the submitted work.

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