DNA methylation study of Huntington’s disease and motor progression in patients and in animal models

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Although Huntington’s disease (HD) is a well studied Mendelian genetic disorder, less is known about its associated epigenetic changes. Here, we characterize DNA methylation levels in six different tissues from 3 species: a mouse huntingtin (Htt) gene knock-in model, a transgenic HTT sheep model, and humans. Our epigenome-wide association study (EWAS) of human blood reveals that HD mutation status is significantly (p < 10^-7) associated with 33 CpG sites, including the HTT gene (p = 6.5 × 10^-26). These Htt/HTT associations were replicated in the Q175 Htt knock-in mouse model (p = 6.0 × 10^-8) and in the transgenic sheep model (p = 2.4 × 10^-88). We define a measure of HD motor score progression among manifest HD cases based on multiple clinical assessments. EWAS of motor progression in manifest HD cases exhibits significant (p < 10^-7) associations with methylation levels at three loci: near PEX14 (p = 9.3 × 10^-9), GRIK4 (p = 3.0 × 10^-8), and COX4I2 (p = 6.5 × 10^-8). We conclude that HD is accompanied by profound changes of DNA methylation levels in three mammalian species.
Huntington’s disease (HD) is a dominantly inherited neurodegenerative disorder clinically characterized by a progressively degenerative, motor, cognitive, and psychiatric impairment. HD gene-expansion carriers (HDGECs) have CAG-repeat lengths of 36 or greater on one of the alleles of the huntingtin (HTT) gene. HD is one of several polyglutamine disorders (including spinal cerebellar ataxias types 1, 2, 3, 6, and 7, spinal and bulbar muscular atrophy, and dentatorubral-pallidoluysian atrophy) that are caused by the expansion of unstable CAG trinucleotide repeats.

Since these disorders exhibit distinct patterns of neuronal loss and clinical manifestation, the differential pathogenesis of polyglutamine disorders may be due to differences in polyglutamine protein context or the host protein function. This is despite nearly ubiquitous expression of these proteins, at least in the brain, and, in the case of HTT, expression throughout the body and during development. The age of onset of motor (AMO) symptoms is strongly and inversely correlated with the number of CAG trinucleotide repeats in HTT.

A recent study showed that HTT CAG-repeat length but not polyglutamine length determines the timing of HD motor onset. HD patients are usually clinically diagnosed in their 40s, but the AMO ranges from younger than 10 years for individuals with high-repeat lengths to, in rare cases, over 80 years. Age plays an important role in HD; for example, the product of CAG-repeat length and chronological age (CAP score) relates to clinical progression in HD according to longitudinal studies of HDGEC cohorts.

By exploiting DNA methylation (DNAm)-based biomarker of tissue age (referred to as the epigenetic clock), we have shown that HD is associated with epigenetic age acceleration (AgeAccel) and greatly disrupted changes in DNAm levels in brain tissues. Recent studies have looked at methylation levels of selected genes in HDGECs and analyzed 13 human cortical samples. DNA (de)methylation in an HTT gene context has also been investigated in transgenic animal models. However, it is not yet known whether HD is associated with significant DNAm changes in other human tissues and in transgenic animal models.

Here we present, to our knowledge, the largest DNAm study of HD to date. We generate large methylation datasets from three species and from eight sources of DNA. We evaluate composite epigenetic biomarkers and individual CpGs in terms of their relationship with (a) HDGEC status and (b) HD progression in individuals with manifest motor symptoms. Our epigenetic clock study demonstrates that manifest HD is associated with increased epigenetic age in human blood DNA. Epigenome-wide association studies (EWAS) of HD mutation status reveal that HTT is the most significant locus in multiple tissues from three species. Blood DNAm levels of select genomic loci are associated with motor score progression among manifest HD patients.

Results

Our methylation study datasets derive from three species: (I) a total of 2164 human samples across blood, lymphoblast, and fibroblast tissues collected from five datasets, (II) a total of 112 mouse tissues (cerebellum, striatum, blood, cortex, and liver) measured on two platforms (reduced representation bisulfite sequencing (RRBS) and a custom methylation array), and (III) 168 blood samples from sheep. All methylation datasets are described in Table 1 (for human) and Supplementary Table 1 (for mouse and sheep). Human blood samples were a subset of a larger collection from Enroll-HD and Registry-HD datasets; the longitudinal clinical measures from the subset of cases were used to perform the HD progression analysis.

Human data. We generated five human DNAm datasets using the Illumina Infinium array (N = 2164 samples; Table 1): (1) N = 910 blood samples from Enroll-HD study (data 1); (2) N = 714 longitudinal blood samples from 357 individuals from Enroll-HD (data 2); (3) N = 376 blood samples from Registry-HD; (4) N = 100 lymphoblastoid cell samples from Registry-HD; and (5) N = 64 fibroblast samples from the CHDI. The Enroll-HD study datasets (blood data 1 and 2) used in this study were a subset of a larger collection of 33,288 observations from 15,203 individuals (“Methods”; Supplementary Tables 2, 3). Apart from studying the effect of HTT mutation status on DNAm levels, we also tested the hypothesis that methylation levels in blood-derived DNA are predictive of motor progression in manifest HD cases. We analyzed large-scale longitudinal motor score assessments from (1) Enroll-HD data 1 after the initial blood draw (on average 3.1 years of follow-up; Supplementary Table 2), (2) the Enroll-HD data 2, which only involved HDGECs (on average 7.9 years apart from the first blood draw; Supplementary Table 3), and (3) the last visit in the Registry–HD cohort (Table 1). Our EWAS of HD progression involved 917 individuals with manifest HD for whom both DNAm profiles and longitudinal clinical assessments were available (“Methods”).

HD motor progression in manifest HD cases. A linear mixed analysis was performed to define a measure of HD motor progression in manifest patients from Enroll-HD. For data 1, we used a large-scale Enroll-HD database comprising 14,850 longitudinal observations across 5204 manifest patients (“Methods”). Total motor scores were measured by the Unified Huntington’s Disease Rating Scale (UHDRS)12, a standard clinical assessment to quantify the severity of disease (Supplementary Note 1). A higher UHDRS motor score indicates a more severe disease progression. Figure 1a illustrates the heterogeneity of slopes of longitudinal motor scores versus visit, indicating the utility of modeling visit as a random effect in a linear mixed-effects model. A linear mixed-effects model was used to relate the longitudinal motor scores (dependent variable) to visit (random effect), age, sex, CAG length, age at motor onset, and educational attainment (“Methods”). Our measure of HD progression was defined by the random slope estimate after adjusting for the other covariates. The adjusted random slope estimate can be viewed as a measure of atypical HD progression not predicted by HTT mutation, age, and potential confounders. This measure is analogous to the one used in an earlier genome-wide association study (GWAS) of HD progression13.

After >2 years of follow-up, motor scores were substantially increased (on average 7.6 points; Supplementary Fig. 1). As expected, CAG-repeat length and AMO were significantly associated with an increase in motor score in a linear mixed model analysis (Table 2). The model revealed that motor scores were increased by 2.7 ± 0.09 (p = 5.3 × 10−191) per one unit increase in CAG-repeat length, increased by 2.2 ± 0.05 (p < 5 × 10−300) per year (of age), increased by 3.5 ± 0.08 (p < 5 × 10−300) per year of follow-up, and decreased by 1.54 ± 0.05 (p = 1.24 × 10−201) per year of AMO. Females were associated with higher motor scores (p = 1.16 × 10−12) and years of education were associated with lower motor scores (p = 3.31 × 10−22).

A linear mixed analysis was analogously performed in Enroll-HD data 2 (1867 observations across N = 278 patients, with a longer follow-up period (“Methods” and Supplementary Table 4). The motor score in this group at the last visit showed a substantially larger increase (on average 27.43 compared to 7.6), due to the longer follow-up period (on average 7.8 years compared to 2 years; Supplementary Fig. 1). Both linear mixed-effects models reveal that our measures of HD motor progression (random slope) are significantly associated with age and CAG-repeat length in both Enroll-HD data 1 and 2 (Table 2 and
HD manifest patients exhibiting accelerated blood epigenetic aging effects. We applied five human epigenetic biomarkers of aging (epigenetic clocks) that lend themselves to analyzing blood methylation data: (1) Horvath’s pan-tissue epigenetic age (referred to as DNAm age \(^{14}\)); (2) Hannum’s blood-based DNAm age \(^{15}\); (3) skin and blood clock (DNAmAgeSkinClock)\(^{16}\); (4) DNAmPhenome-Age\(^{17}\); and (5) the mortality risk estimator DNAmGrimAge\(^{18}\).

Using the longitudinal Enroll-HD data 2, we found that all DNAm age estimators track the passage of study time (Fig. 2a–d). In the cross-sectional data, the correlation between DNAm age estimators and chronological ages were >0.84, led by DNAmAgeSkinClock (Fig. 2c) with correlation estimates ranging between 0.95 and 0.97 across all the three datasets (Supplementary Figs. 2e, f, 3c, and 4c).

To formally measure possible epigenetic AgeAccel effects due to HD disease status, we fit a regression model of DNAm age on chronological age and defined AgeAccel as the resulting raw residual. Thus, positive AgeAccel means the methylation state of the sample appears to be older than would be expected based on chronological age. We found that manifest HD (but not pre-manifest HD) had significantly higher epigenetic AgeAccel than controls (0.098 \(\leq p \leq 2.0 \times 10^{-4}\), Fig. 3), led by the pan-tissue clock (AgeAccel, \(p = 2.0 \times 10^{-4}\), Fig. 3a) and AgeAccelGrim (\(p = 2.4 \times 10^{-4}\), Fig. 3b). The difference remained significant (\(p = 1.1 \times 10^{-3}\); Fig. 3b) when studying the measure of intrinsic epigenetic AgeAccel (IEAA), which is independent of imputed blood cell counts\(^{19}\). Despite the low number of controls (\(N = 78\)), we managed to corroborate the finding for AgeAccelGrim in the Registry-HD data (\(p = 0.046\); Supplementary Fig. 5L).

A multivariate model analysis demonstrates that the observed epigenetic AgeAccel effects are not due to confounders (Supplementary Table 6). In particular, we find that manifest HD is associated with a significant increase in DNAm age (\(p = 2.9 \times 10^{-7}\) in the Enroll-HD data even after adjusting for the 10 principal components. However, this association could not be replicated in the Registry-HD data (Supplementary Table 7).

The pan-tissue clock also revealed a strong correlation between lymphoblastoid cells blood (\(r = 0.74, p = 2.1 \times 10^{-18}\); Supplementary Fig. 6A), but the DNAm Age estimate was significantly lower than that of corresponding blood samples (mean difference = 3.3 years, \(p = 0.0017\); Supplementary Fig. 6B), suggesting that the Epstein–Barr virus transformation used in generating lymphoblastoid cell lines decreases DNAm age.

Next, we tested whether epigenetic AgeAccel is associated with motor progression in manifest HD. The considered measures
**Fig. 1** EWAS of motor progression in manifest HD cases. **a** The spaghetti plot illustrates how the motor score depends on the visit in Enroll-HD. To arrive at a measure of motor score progression (i.e., the rate of change in the motor score), we estimated the slope using a random-effects model with a random slope and intercept term. **b** The Manhattan plot visualizes the EWAS results of motor progression in manifest HD cases: log (base 10)-transformed meta-analysis p value (y-axis) versus the chromosomal location of each CpG (x-axis). The EWAS results were calculated with the biweight midcorrelation tests. Fixed-effects meta-analysis (weighted by inverse variance) was performed to combine the EWAS results across blood data (N = 917) from Enroll-HD data 1, Enroll-HD data 2, and Registry data. All p values are two-sided and not adjusted for multiple comparisons. The blue and red horizontal lines correspond to suggestive significance (\( \alpha = 1.0 \times 10^{-5} \)) and genome-wide significant levels (\( \alpha = 1.0 \times 10^{-7} \)), respectively. Gene names are provided for CpGs (blue circles) with p < 1.0 × 10^{-6} with detailed summary statistics reported in Supplementary Data 3.

### Table 2 Linear mixed model regression analysis for HD motor progression.

| Parameter                  | Outcome: motor score | Outcome: random slope |
|----------------------------|----------------------|-----------------------|
|                            | Coef. | SE    | T statistic | P value | Coef. | SE    | T statistic | P value |
| Female                     | 3.375 | 0.474 | 7.127       | 1.16 × 10^{-12} | 0.016 | 0.051 | 0.316 | 0.8 |
| Age                        | 2.217 | 0.045 | 48.878      | <5.0 × 10^{-300} | 0.013 | 0.005 | 2.749 | 5.99 × 10^{-3} |
| CAG length                 | 2.743 | 0.089 | 30.757      | 5.27 × 10^{-191} | 0.071 | 0.010 | 7.449 | 1.09 × 10^{-13} |
| Age at motor onset         | -1.542| 0.049 | -31.687     | 1.24 × 10^{-201} | -0.010 | 0.005 | -2.006 | 4.49 × 10^{-2} |
| Education                  | -1.877| 0.193 | -9.742      | 3.09 × 10^{-22}  | -0.083 | 0.021 | -4.037 | 5.49 × 10^{-5} |
| Visit                      | 3.516 | 0.078 | 45.019      | <5.0 × 10^{-300} |      |      |      |      |

The table presents the coefficient estimates from two linear regression models: (1) a linear mixed model analysis of 14,850 longitudinal motor scores (dependent variable) across 5204 manifest HD cases from Enroll-HD data 1 and (2) a linear regression model analysis of the resulting random slope estimates (dependent variable) of the same 5204 cases. The linear mixed-effects model included two random-effects (a random intercept term and a random slope term with respect to visit) and several fixed-effect terms (sex, age at baseline, CAG-repeat length, age at motor onset, educational attainment). The empirical Bayes estimate of the random slope was used as a measure of HD motor progression for each of the N = 5204 manifest HD cases. We adjusted the random slope estimate for all fixed effects in our downstream EWAS analysis. The columns report the covariate name, regression coefficient, standard error, Student’s T statistic, and unadjusted two-sided Wald test p value.
of epigenetic AgeAccel exhibit nominally significant positive correlations with motor progression (e.g., $r = 0.08$, meta-analysis $p = 0.016$ for the pan-tissue clock; Supplementary Table 8 and Supplementary Fig. 7).

**Human EWAS of HDGEC status.** In our EWAS, we related HDGEC status to individual epigenetic markers (CpGs) using Enroll-HD data 1 and Registry-HD, then combined the results using the Stouffer’s method. To avoid technical confounding by chip effects in the Enroll-HD data 1, we used the aggregated Enroll-HD data for our EWAS analysis (Supplementary Table 9). The association between HD and individual methylation levels is strongly preserved across the two datasets ($r = 0.31$; Supplementary Fig. 8). The Manhattan plot reveals that CpG cg22982173 in exon 1 of the $HTT$ gene on chromosome 4 was most significantly associated with HD status (Stouffer’s $p = 6.5 \times 10^{-26}$; Fig. 4a and Supplementary Fig. 9).

We set up a significance threshold at $\alpha = 10^{-7}$ to adequately control for false-positive rate in the DNAm array studies. We found 33 genome-wide significant CpGs for HD status (Table 3 and Supplementary Data 1), including cg22982173 in $HTT$ ($p = 7.0 \times 10^{-26}$), cg26892702 near DVL2 ($p = 5.3 \times 10^{-10}$), cg23819669 near DNAJB8 ($p = 4.2 \times 10^{-9}$), cg19759282 near GPR155 ($p = 4.4 \times 10^{-9}$), cg16739503 near RASA3 ($p = 6.5 \times 10^{-9}$), cg20684718 near MCM10 ($p = 1.4 \times 10^{-8}$), cg22296756 near HDAC4 ($p = 6.9 \times 10^{-8}$), and probe ch.3.75336R near MLH1 ($p = 2.4 \times 10^{-8}$).

**HTT methylation across multiple brain regions.** We studied $HTT$ cg22982173 using 475 brain methylation samples from our previous study (26 HD versus 39 control individuals; "Methods")6. Figure 5 lists the bar plots for the seven selected brain regions, with right temporal cortex ($p = 0.02$; Fig. 5e) and midbrain ($p = 0.046$; Fig. 5h) reaching statistical significance for increased methylation at the $HTT$ cg22982173 locus in HD cases compared to controls. Methylation levels of cg22982173 across all brain regions combined were significantly higher in the HD group (Fig. 5a: $p = 5.6 \times 10^{-6}$ and remained significant, $p = 1.5 \times 10^{-2}$, after adjusting for intra-subject dependence).

Our top EWAS hit in the blood (in the $HTT$ locus) continues to be associated with HD status in several brain regions (Fig. 5), while the EWAS results in brain tissue are different from those of blood tissue on a global level (Supplementary Fig. 13). Supplementary Figures 11 and 12 report bar plots of cg22982173 stratified by study and tissue types: blood, lymphoblastoid cell, fibroblast, and a total of 14 brain regions. The CpG cg22982173 resides within a region of the genome with a high density of CpGs (i.e., in a CpG island) and therefore would be expected to have low levels of methylation; however, significant increases in methylation were observed in HD cases.

**Both CAG-repeat alleles correlate with human $HTT$ methylation.** CAG-repeat lengths were assessed for both long and short alleles (CAG.long and CAG.short) in the human $HTT$ exon 1 locus. We correlated both alleles to human $HTT$ methylation (at cg22982173) using all $N = 2164$ human samples. A series of
multivariate regression models were tested to evaluate the joint effect of CAG.long and CAG.short in (i) all, (ii) controls only, and (iii) HD mutation carriers only, respectively (“Methods”).

Strikingly, our comprehensive model fitting analysis in different data and cell types demonstrated that both CAG.long and CAG.short were independently associated with HTT methylation (Supplementary Data 2). These significant associations were observed even after restricting the analysis to controls or to HD mutation carriers, respectively. Interestingly, the product of the two CAG-repeat lengths (CAG.product = CAG.long × CAG.short) showed the best relationship with HTT methylation according to the Bayesian information criterion (BIC) (Supplementary Data 2). The CAG.product model exhibited the best fit (lowest BIC value) when considering all \( N = 910 \) blood samples from the Enroll-HD data 1, all \( N = 376 \) blood samples from the Registry-HD, \( N = 294 \) controls from Enroll-HD data 1, \( N = 616 \) HD mutation carriers from Enroll-HD data 1, the \( N = 298 \) HD mutation carriers from Registry-HD, \( N = 37 \) mutation carriers from the lymphoblastoid data, and \( N = 37 \) mutation carriers from the fibroblast data.

The quadratic term in CAG.long was also significantly related to HTT methylation using polynomial modeling analysis. The multivariate linear models showed that age and sex only have negligible effects on the methylation levels of cg22982173 (Supplementary Data 2).

The moderately high correlation estimates \( (r = 0.4–0.65) \) between the CAG.product and HTT methylation levels were replicated in several datasets (meta-analysis \( p \) value of \( 9.1 \times 10^{-123} \); Fig. 6). Significant correlations can be observed even when using only controls (meta-analysis \( p = 4.5 \times 10^{-8} \)) or only HD mutation carriers \( (p = 3.9 \times 10^{-27}; \) Supplementary Figs. 14 and 15).

The methylation levels of cg22982173 were low both in HD cases and controls: the \( \beta \) value (interpreted as the proportion of chromosome methylated at this locus) ranged from 0.015 to 0.063 (Supplementary Fig. 16).
Human \textit{HTT} methylation is not confounded by other trinucleotide tandem repeats. It is well known that the HD expansion is overrepresented on a subset of the haplotypes associated with non-disease-associated alleles. Notably, a potential methylation-modifying polymorphism exists in the form of the polymorphic CCG repeat, which lies immediately downstream of the CAG repeat and tags many of the most common \textit{HTT} haplotypes.

We evaluated five multivariate linear regression models to examine if the association between \textit{HTT} and CAG length could possibly arise as an artifact of linkage disequilibrium between the CAG repeat and some other methylation-modifying variants at the \textit{HTT} locus (“Methods”). Especially, we examined the model (Model 5) that omitted all HD cases that carried an atypical \textit{HTT} structure, defined as those that do not conform to the “typical” reference alleles (CAG)$_{12}$CAACAGCCG(CCG)$_{2}$CCT (where Q1 and P2 are numbers of pure CAGs and CCGs, respectively)\(^2\). We studied 372 individuals from the Registry-HD cohort, which revealed a marked association between CAG- and CCG-repeat lengths in the Registry-HD cohort ($r^2 = 0.16$, $p < 2 \times 10^{-16}$; Supplementary Fig. 17A). However, several lines of evidence suggest that the most associations we have observed in manifest HD cases, we performed a meta-analysis that combined three separate EWAS of (adjusted) motor progression using the two Enroll-HD and the Registry-HD datasets, respectively (“Methods”). Interestingly, higher methylation of the \textit{HTT} locus (cg22982173) was associated with slower HD progression in manifest HD cases according to the motor score ($p = 0.05$), Stroop tests for cognitive function assessment (color naming $p = 0.01$, word reading $p = 0.07$, and interference test $p = 0.06$), and functional assessment score ($p = 0.009$).

Our blood EWAS of HD progression in manifest HD cases found three genome-wide significant CpGs: cg26919387 (meta-analysis $p = 9.3 \times 10^{-9}$) in PEX14, cg12823408 near GRIK4 (meta-analysis $p = 3.0 \times 10^{-8}$), and cg21497164 in COX4I2 (meta-analysis $p = 6.5 \times 10^{-8}$; Fig. 1b, Supplementary Fig. 18, Supplementary Table 11, and Supplementary Data 3). The robust correlation (approximate $r = -0.19$) indicated that all three CpGs that were hypomethylated were associated with HD motor progression.

The top EWAS hits were not confounded by single-nucleotide polymorphisms (SNPs). CpG measurement can be compromised by neighboring SNPs. We addressed this concern using two approaches. First, we correlated the significant CpGs with SNPs that located near the CpG probes according to the Illumina annotation file. No significant correlations were observed. Second, we applied the software Gaphunter\(^2\)3 and found only 1 CpG (cg04285477 near Cl4orf43) out of 33 EWAS CpGs associated
with HD disease status exhibited weak evidence of confounding (i.e., a clustering pattern; “Methods”). None of the three CpGs (top hits) associated with motor progression showed evidence of confounding.

**EWAS of motor progression was not confounded by treatment.**

Tetrabenazine, a medication for symptomatic treatment of chorea, was used by ~23% of HD patients in Enroll-HD. Treated and untreated HD patients did not differ in terms of age, but treated patients exhibited a higher CAP score (p = 0.01 in Enroll-HD data and p = 8.2 × 10^{-8} in Enroll-HD data 2) and a higher motor score (p = 3.2 × 10^{-4} in Enroll-HD data 1 and p = 2.1 × 10^{-9} in Enroll-HD data 2; Supplementary Figs. 19 and 20). Tetrabenazine was not significantly associated with any of the three motor progression-associated CpGs as depicted in Supplementary Fig. 21. Furthermore, no CpGs on the Illumina array were associated with tetrabenazine treatment at a genome-wide significance level of p = 1.0 × 10^{-7} (Supplementary Fig. 22). Overall, these results demonstrate that the EWAS of HD progression is not confounded by tetrabenazine treatment.

**Methylation data from Htt knock-in mice (Q175).** We generated two types of DNAm datasets (RRBS and custom methylation array) from a heterozygous (HET) Htt knock-in (KI) mouse model (Q175 versus Q20 CAG repeats or wild-type mice; “Methods”).

We conducted EWAS of mutant HTT gene (Q20 versus Q175) across the striatum and cerebellum in RRBS data. Focusing on CpGs in the murine Htt locus revealed a moderately strong correlation between the striatum and the cerebellum (r = 0.55 and p = 5.3 × 10^{-11}; Supplementary Fig. 23). Strikingly, CpG sites within the Htt locus are among the top EWAS results for Q175 status in murine striatum and cerebellum (Supplementary Fig. 24). This finding is also illustrated by our meta-analysis EWAS across the striatum and cerebellum (Fig. 4b). Our EWAS identified two genome-wide significant CpGs in Htt region, chr5:34762314 (meta-analysis p = 6.0 × 10^{-8}) and chr5:34762241 (p = 9.2 × 10^{-8}) located between exons 1 and 2 (Fig. 4b, d and Supplementary Data 1). These CpGs were located ~1 kb in the 3’ direction of Htt exon1 (Fig. 4d), both were hypermethylated in the Q175 group.

Analyzing the same brain regions from another group of mice using our custom array platform revealed another highly significant CpG to be hypomethylated in the Q175 group (probe cg12389415 on the custom array, mouse chr5:34795955) also within the Htt locus (meta-analysis p = 3.2 × 10^{-22}; Supplementary Fig. 25). Integrating the EWAS results across all the available tissue types: the two brain regions, blood, cortex, and liver, increased the statistical significance of the association (Stouffer meta-analysis p = 6.2 × 10^{-43}). The Htt gene locus was the top

| Chr. | CpG | bp   | CpG island | Gene | Gene location | Meta p | Meta Z |
|------|-----|------|------------|------|---------------|--------|--------|
| 4    | cg22982173 | 3,076,557 | Island | HTT | First exon | 6.5 × 10^{-26} | 10.53 |
| 17   | cg26892702 | 7,132,489  | Island | DL2 | Body | 5.3 × 10^{-10} | -6.21 |
| 3    | cg23819669 | 128,187,274 | Island | DNAIB8 | TSS1500 | 4.2 × 10^{-9} | -5.88 |
| 2    | cg19759282 | 175,351,649 | Island | GPRISS | 5’-UTR; 1st exon | 4.4 × 10^{-9} | 5.87 |
| 13   | cg16739503 | 114,862,324 | Island | RASA3 | Body | 6.5 × 10^{-9} | -5.8 |
| 10   | cg20687418 | 13,248,241  | Island | MCM10 | Body | 1.4 × 10^{-8} | -5.68 |
| 12   | cg04650980 | 117,471,700 | Island | MEF2 | Body | 1.8 × 10^{-8} | -5.63 |
| 11   | cg06993329 | 886,695     | N_Shore | CHD1 | Body | 1.9 × 10^{-8} | -5.63 |
| 17   | cg04683330 | 80,041,239  | Island | FASN | Body | 1.9 × 10^{-8} | -5.62 |
| 12   | cg16320626 | 121,877,851 | Island | KDM4B | Body | 2.1 × 10^{-8} | -5.6 |
| 3    | cg37336232 | 37,048,044  | Island | HMLH1 | 5’-UTR; body | 2.4 × 10^{-8} | 5.58 |
| 18   | cg09080920 | 928,338     | Island | LMX1 | Body | 2.5 × 10^{-8} | -5.57 |
| 22   | cg14703589 | 31,644,558  | Island | LIFK2 | First exon; 5’-UTR; body | 2.6 × 10^{-8} | -5.57 |
| 12   | cg17859634 | 124,798,815 | Island | FAM101A | Body | 3.4 × 10^{-8} | -5.52 |
| 14   | cg04285477 | 74,194,516  | Island | CI4orf43 | Body | 3.6 × 10^{-8} | -5.51 |
| 17   | cg23148992 | 3,558,246   | Island | CTS5 | Body | 4.1 × 10^{-8} | -5.49 |
| 19   | cg08117431 | 5,455,497   | Island | ZNF94 | 5’-UTR; 1st exon | 4.2 × 10^{-8} | -5.48 |
| 2    | cg15115604 | 241,069,379 | Island | MYEOV2 | Body | 5.0 × 10^{-8} | -5.45 |
| 14   | cg10929299 | 50,334,860  | Island | DDX39 | TSS200 | 5.6 × 10^{-8} | -5.43 |
| 19   | cg10588570 | 14,530,314  | Island | BTBD10 | TSS1500 | 6.2 × 10^{-8} | -5.4 |
| 3    | cg15746396 | 13,458,203  | Island | POLR2J4 | TSS200 | 6.9 × 10^{-8} | -5.39 |
| 7    | cg22296476 | 240,305,369 | Island | HDAC4 | 5’-UTR | 6.9 × 10^{-8} | -5.39 |
| 19   | cg1543665 | 8,373,294   | Island | CD320 | TSS201 | 6.9 × 10^{-8} | -5.39 |
| 2    | cg05054944 | 25,856,333  | Island | DNB | Body | 7.2 × 10^{-8} | -5.39 |
| 16   | cg00284420 | 87,311,948  | Island | N_Shore | ZNF94 | 7.5 × 10^{-8} | -5.38 |
| 17   | cg24965497 | 7,227,181   | Island | ZNF94 | 5’-UTR; 1st exon | 7.6 × 10^{-8} | -5.38 |
| 2    | cg13731523 | 3,047,190   | Island | S_Shelf | SEPW1 | 8.0 × 10^{-8} | -5.37 |
| 17   | cg14517001 | 80,179,931  | Island | SEPW1 | TSS1500 | 8.4 × 10^{-8} | -5.36 |
| 16   | cg20635595 | 28,481,627  | Island | PDE3B | Body | 8.7 × 10^{-8} | -5.35 |
| 11   | cg16356630 | 14,683,035  | Island | STBD1 | First exon; 5’-UTR | 9.0 × 10^{-8} | -5.35 |
| 12   | cg1881599 | 92,814,084  | Island | CULLI1OS | 3’-UTR; TSS1500 | 9.2 × 10^{-8} | -5.34 |

The table reports 33 genome-wide significant CpGs (meta-analysis p value < 10^{-7}). A positive (negative) value of the meta-analysis Z statistic indicates that the CpG is hypermethylated (hypomethylated) in human HDGECs. In this case-control study, we did not distinguish pre-manifest from manifest HD. The Z statistic is based on Stouffer’s meta-analysis method across the aggregated Enroll-HD samples and the Registry-HD data. Columns report the chromosome, CpG position (hg19 assembly), the relative location to the nearest CpG island, nearest gene, location of the CpG with respect to the gene, meta-analysis (unadjusted, two-sided) p value, and Z statistic.

*The unusual probe name “ch.3.753362R” reflects a CpH probe (CA, CC, CT) as opposed to a CpG probe, which targets the reverse strand on chromosome 3 (hg19 coordinate 753362).
Correlations using a linear mixed-effects model. The cortex, left frontal cortex, caudate nucleus, and midbrain, respectively. The blue dots correspond to individual observations that underlie the respective mean values. The bar. The heading of each panel reports a nominal (unadjusted) two-sided CAG expansion in exon 1 of that significant across three species, human, mouse and sheep (Fig. 4d), revealed...1.8 × 10−13). The top 1000 hypomethylated CpGs were adjacent to genes (hypergeometric p = 3.6 × 10−13) implicated by a protein–protein interaction network analysis of polyglutamine disorders (Supplementary Table 14). The most significant enrichment according to the GOMETH analysis involved the

EWAS hit in all five tissues profiled on the custom methylation array (Supplementary Data 1).

Ovine EWAS of HD status. In addition to mice, we also studied a transgenic model of sheep with a human HTT cDNA transgenic sequence24–28. The transgene is integrated into a different location than the endogenous sheep HTT gene. Using a custom methylation array, we generated blood methylation data from 168 sheep: 84 HD transgenic sheep and age-matched controls; Methods). EWAS of HD transgenic status identified five genome-wide significant (p < 1.0 × 10−7) CpGs located on chromosome 10 including one CpG near the HTT transgene (Fig. 4c, according to oviAr4 assembly coordinates 1826–1903 bp). The most significant association was the CpG located in exon 4 of the HTT transgene with a striking p value of 2.4 × 10−88, followed by another CpG in the FLTI gene (p = 1.8 × 10−10, Supplementary Data 1).

Only CpGs close to the CAG expansion are hypermethylated in HD. A comparison of the significant CpGs in the HTT locus across three species, human, mouse and sheep (Fig. 4d), revealed that significant CpGs for HD status are located within 2 kb of the CAG expansion in exon 1 of HTT/Htt. The comparison between human and sheep highlighted that the proximity between a CpG dinucleotide and the location of the CAG expansion in either the endogenous HTT or transgenic HTT (typically <2 kb) plays an important role (Fig. 4d). This is based on the following observations: (a) all genome-wide significant CpGs near the HTT locus are located within 2 kb of the CAG expansion, (b) unlike in humans, HTT exon 4 is close to the site of the CAG expansion in the sheep because the HTT transgene is a cDNA without any intronic genomic DNA, (c) unlike in humans, methylation at a specific CpG in exon 4 of an HTT cDNA fragment relates strongly to HD transgenic gene status in blood samples from the sheep (Supplementary Data 1).

Next, we investigated if HD patients tended to gain methylation in associated HTT CpGs. Interrogating the genomic regions surrounding HTT exon 1, all four CpGs were significantly hypermethylated in HD patients (Supplementary Data 4). Interestingly, all the 27 significant CpGs in Htt (meta-analysis p < 0.05) also exhibited the same hypermethylation pattern in Q175 mice (Supplementary Data 4).

Enrichment analysis of EWAS results. To gain insights on the biological function associated with HD related CpGs, we used two different approaches for carrying out functional enrichment analysis: (1) the anRichment approach under HDinHD29 and (2) GOMETH enrichment analysis, which takes into account the different number of probes per gene present on the Illumina 450k array30. We studied the top 1000 CpGs that were hypomethylated in HD and another set of top 1000 CpGs that were hypermethylated in HD using the anRichment analysis (Supplementary Tables 12 and 13). The top 1000 hypomethylated CpGs were adjacent to genes (hypergeometric p = 3.6 × 10−13) implicated by a protein–protein interaction network analysis of polyglutamine disorders (Supplementary Table 14). The most significant enrichment according to the GOMETH analysis involved the
overlap between the top 1000 hypermethylated CpGs and RNA binding \((p = 3.4 \times 10^{-6};\) Supplementary Table 15).

We carried out an enrichment analysis of CpGs associated with HD progression using the same two approaches. The anRichment analysis identified the HD progression related genes enriched in several known HD relevant gene sets (Supplementary Table 16) and the GOMETH analysis identified gene sets enriched in immune cell activation (Supplementary Table 17).

Discussion

This is by far the largest DNAm study of HD to date. We generated large methylation datasets from seven sources of DNA across three species (human: 2000 blood, 100 lymphoblastoid cells, and 64 fibroblasts; mouse: 32 striatum, 32 cerebellum, 16 liver, 16 cortex, and 16 blood; sheep: 168 in the blood). One of our major findings is that manifest (but not pre-manifest) HD cases exhibit accelerated epigenetic aging effects in blood, which is consistent with the epigenetic AgeAccel observed in various brain regions\(^6\). HD progression in manifest individuals was only weakly linked to epigenetic AgeAccel in blood.

A second major finding of our study was that EWAS (as opposed to GWAS) of the HTT mutation status consistently found CpGs in the HTT locus in all species and tissues. If nothing was known about the genetics of HD, then our human blood methylation data would have implicated the pertinent disease gene: a very significant CpG probe located in HTT exon 1. The HTT methylation effect was validated across seven sources of DNA (blood, lymphoblastoid cells, fibroblasts, striatum, cerebellum, cortex, and liver) and across three species. Our most significant human HTT cg22982173 was neither confounded by neighboring trinucleotide tandem repeats nor dominated by atypical HTT structure. We also analyzed custom methylation array data (providing >1000× coverage per CpG) to address concerns surrounding low sequence coverage (minimum 15× coverage) in the mouse RRBS data\(^31\). Both mouse RRBS and custom methylation array data implicated the Htt gene. A comparison of the significant CpGs in the HTT/Htt locus across three species—human, mouse, and sheep—revealed that significant CpGs for HD status were located in the vicinity of the CAG expansion in exon 1 of HTT/Htt. The methylation levels of cg22982173 exhibited only weak (but nominally significant) negative correlations with measures of motor progression and other clinical assessments (e.g., Stroop test) in manifest HD after adjusting CAG expansion, and other potential confounders. An intriguing and yet unexplained finding, based on our comprehensive model fitting analysis of the most significant CpG cg22982173 in the human HTT locus, was that the methylation level at this locus correlated with the allelic product of CAG lengths in both cases and controls, inferring that methylation at this locus was related to the length of the non-mutated allele as well. Our analysis of HTT methylation in human postmortem brain samples was consistent with that observed in blood samples.

Abundant caution needs to be exercised in linking Htt exon1 methylation to expression levels of the mutant HTT/Htt gene, since CpGs often have only weak effects on neighboring gene expression levels\(^32\). However, it is interesting that at least in the murine Htt allelic series KI mouse model, there is a robust Htt CAG-length-dependent reduction of mutant Htt RNA and protein levels in the striatum\(^29\). Caution is also warranted when interpreting the relationship between transgene expression and CpG methylation in the transgenic sheep model for the following
Our finding on HTT/Htt locus methylation, especially identifying that the most significant HD-associated methylation sites were in close proximity to the repeat expansion mutation itself, may have general implications for understanding the role of epigenetics in repeat expansion disorders. First, similar findings of pathologically relevant abnormal DNAm in the proximity of expanded pathogenic repeats have been observed in other disorders such as fragile X syndrome, myotonic dystrophy type I, and Friedreich’s ataxia. Second, a recent study showed 22 disease-associated tandem repeats, including those found within gene: FMR1 (gene mutated in fragile X), frataxin, HTT, ataxin 1, and ataxin 3 were located in chromatin domain boundaries enriched with an ultra-high density of CpG islands and bound by methylation-sensitive chromatin factor CTCF. 

Another important area of investigation is whether methylation changes on HTT exon 1 have any impact on the somatic instability of the mutant CAG repeat itself. Recent large-scale human genetic studies revealed variants in mismatch repair enzymes (e.g., MLH1, MSH2, MSH3), which are known to modify the germline and somatic instability of expanded HTT CAG repeats in KI mouse models, act as modifiers of the age of motor symptom onset in HD. Our EWAS of HDGEC status in human blood identified 33 loci at a genome-wide significance level. Besides HTT itself, the EWAS revealed the following genes known to modify mutant HTT aggregation or toxicities. DNAJ8 is a member of the DNAJ (Hsp40) chaperone family and inhibits polyglutamine mutant HTT aggregation and amyloid fibril formation. HDAC4 binds to mutant HTT in a polyglutamine length-dependent manner and is localized to the cytoplasmic inclusions in striatal neurons in HD mouse models, and genetic reduction of murine Hdaac4 reduces cytoplasmic mutant Htt aggregation and restores cortico-striatal synaptic transmission in HdhQ150 KI mice. Another clinically relevant finding determined by blood EWAS of HD motor progression was that epigenetic modifiers were linked to symptom progression in HD. Our analysis focused on progression of motor symptoms in manifest HD cases discovered three CpGs: cg26919387 near PEX14 (p = 9.3 × 10⁻⁹), cg12823408 near GRIK4 (p = 3.0 × 10⁻⁸), and cg21497164 in COX4I2 (p = 6.5 × 10⁻⁸). These findings were not confounded by medications used for treating movement disorders (tetrazenazine). Arguably, the most interesting gene identified was GRIK4, which is selectively expressed in the cortex and striatum. Studies of mice deficient in Grik4 or elevated Grik4 gene dosage have demonstrated the critical role of this molecule in sensory motor gating and protection against excitatory neurotoxicity. 

While our EWAS findings in blood were different to those observed in a previous analysis of brain tissue using a limited brain methylation dataset, we found that several brain regions exhibited hypermethylation of the HTT cg22982173 locus in HD cases. In our two independent murine studies, we observed genome-wide significant hyper- and hypomethylation at the Htt locus in the striatum and cerebellum of Q175 KI mice. Furthermore, our top EWAS hit inside the Htt locus replicated in human lymphoblastoid cells and in fibroblasts.

Overall, our study provides new insights and directions of HD research in the areas of accelerated epigenetic aging, the role of HTT locus methylation and HD biology, and other methylation sites that may act as epigenetic modifiers that modulate HD progression.

Methods

Data sets. HD mutation carriers did not differ significantly from controls in terms of chronological age. However, pre-manifest HD samples tended to be significantly younger than manifest HD samples. Further, the study sample ascertainment induced a strong negative correlation between CAG length and age at enrollment (r = -0.5, p = 2.4 × 10⁻¹⁰ in Enroll-HD). Thus, manifest HD status (or equivalently pre-manifest HD status) was confounded by chronological age in our data. Overall, we observed a significant association between HD disease status and Illumina chip ID in the Enroll-HD study (but not in the Registry-HD study). Most Illumina chips contained 12 manifest HD samples or 12 pre-manifest HD samples or 12 controls. Since chip effects probably confound the relationship between HD status and DNAm levels, we removed the chip effects from the DNAm data by averaging the DNAm levels of each CpG across the 12 samples on a given chip. The HD status of a chip corresponded to the shared HD status of the 12 samples. By replacing individual level data by “aggregated Enroll-HD data,” we effectively removed chip effects at the cost of reduced sample sizes (from N = 910 Enroll-HD samples to 76 corresponding chips). Illumina chips were not confounded by HD status in the Registry-HD study.

Enroll-HD: a prospective registry study in a global HD cohort. We obtained phenotypic data (demographic data and clinical assessments) from the longitudinal Enroll-HD study based on 15,203 individuals. Only a subset of these 15,203 individuals was profiled on the DNAm array: (1) Enroll-HD data 1 were based on a single buffy coat sample from N = 910 individuals (controls, pre-manifest HD, and manifest HD) from Enroll-HD data 1. The Enroll-HD data 2 involved two buffy coat samples (from two separate blood draws) from 368 HTT mutation carriers. The first blood sample from each individual was on average collected 7.9 years before the second sample. We removed 11 of the 368 individuals from the analysis because (a) two individuals were severe outliers according to our epigenetic clock analysis and nine individuals already appeared in the Enroll-HD data 1. Thus, we only studied 357 individuals from the Enroll-HD data 2 study in our analysis. Enroll-HD is a longitudinal, observational, multinational study that will integrate two existing HD registries, Registry-HD in Europe and COHORTH in North America and Australia, while also expanding to include sites in Latin America and Asia. ClinicalTrials.gov Identifier: NCT00757803 (Principal Investigator: Bernhard G. Landwehrmeyer and Study Director: Joseph Giuliano).

With indefinite and ongoing annual assessments, the goal of Enroll-HD is to build a large and rich database of longitudinal clinical information and biospecimens. This database will serve as a basis for future studies aimed at developing tools and biomarkers for progression and prognosis, identifying clinically relevant phenotypic characteristics, and establishing clearly defined endpoints for interventional studies. Core datasets are collected annually on all research participants as part of this multi-center longitudinal observational study of HD. Data are monitored for quality and accuracy using a risk-based monitoring approach. All sites are required to obtain and maintain local Ethics Committee approval.

Enroll-HD is to develop a comprehensive repository of prospective and systematically collected clinical research data (demography, clinical features, family history, genetic characteristics) and biological specimens (blood) from individuals with manifest HD, unaffected individuals known to carry the HD mutation or at risk of carrying the HD mutation, or HDmutation research participants (e.g., spouses, siblings, or offspring of HD mutation carriers known not to carry the HD mutation). Enroll-HD is conceived as a broad-based and long-term project to maximize the efficiencies of non-clinical research and participation in clinical research. With over 200 sites in roughly 30 countries, Enroll-HD is the largest database available for HD researchers. Our Enroll-HD data 1 involved blood samples from Northern America (91%), Europe (12.8%), Latin America (1.1%), and Oceania (5.1%). All individuals were 18 years or older. Both sexes were eligible for the study as were healthy volunteers. A non-probability sampling method was used.

Informed consent from the potential participant or legal representative is a prerequisite for study participation. Participants’ RR consent is an unconditional prerequisite for patient participation in the Enroll-HD, approved by the Scientific Review Committee [https://enroll-hd.org/for-committees/]. More details of the ethics oversight are listed in Enroll-HD protocol [https://www.enroll-hd.org/enrollhd_documents/Enroll-HD-Protocol-1.0.pdf].

In summary, we describe the study, variation from Enroll-HD. Patients with HD and their family members are recruited from specialty clinics (Human Genetics, Neurology, Psychiatry) that advise and treat people affected by HD. In addition, in some areas, community clinics and neurologists who see HD patients will recruit participants for this study. More details for the study population including inclusion and exclusion criteria are listed in Supplementary Note 2.

Registry-HD data. The European Huntington’s Disease Network (EHDN) provided us with whole DNA samples from the Registry-HD core research project. We analyzed 376 samples from whole blood and 100 samples from lymphoblastoid cell
lines. Registry-HD is a multi-center, multinational observational study with no experimental treatment. It forms part of the Huntington Project, a worldwide collaboration dedicated to finding treatments that make a difference for HD. Registry-HD is sponsored by the High Q Foundation, a non-profit organization that supports a variety of research projects seeking to find treatments for HD. The study description of Registry-HD is listed in NIH ClinicalTrials database [https://clinicaltrials.gov/ct2/show/NCT01990899]. Participants IRB consent was obtained at the time of enrollment. A participant visit is an explanation of the study, approved by the Scientific and Bioethical Advisory Committee of the EHDN. Details of the consent are listed the section of “Participant informed consent” of the study protocol [http://www.ehdn.org/wp-content/uploads/2018/06/registry-protocol-2.0.pdf].

Human fibroblasts. We obtained N = 64 fibroblast samples from the CHDI Multiple Tissue Monitoring (MTM) study. The study involved 37 HD mutation carriers and 27 age-matched controls. The mean age was 42.7 years and the proportion of males was 45%. DNAm arrays were profiled in fibroblast tissues. The mean CAG lengths in the HD mutation group were 44 and 19 for the long and short allele, respectively. The CAG lengths were not available in the control group and were set to 28 and 18 for the long and short allele, respectively. The data are described in Table 1.

DNAm data. DNA was extracted from the Registry-HD and the Enroll-HD samples using the Millipore salting out procedure and the QIAGEN QIAamp DNA Midi Kit. DNA was eluted in Buffer AE (a TE buffer). Bisulfitization conversion was performed using the Zymo EZ DNA Methylation Kit (Zymo Research, Orange, CA, USA) as well as subsequent hybridization to the Illumina DNAm array, and scanning (Scan, Illumina) were performed according to the manufacturer’s protocols for technical variations. DNAm array data were profiled from the Illumina Methylation450 Bead Chip for Enroll-HD data 1 and Registry-HD, and profiled on the EPIC array (also known as 850k array) for Enroll-HD data 2. DNAm levels (β values) were determined by calculating the ratio of intensities between methylated (signal A) and unmethylated (signal B) sites. Specifically, the β value was calculated as a ratio of the methylated (M corresponding to signal A) and unmethylated (U corresponding to signal B) sites, as the ratio of fluorescent signals β = Max(M,M0)/(Max(M0) + Max(U,0) + 100). Thus, β values range from 0 (completely unmethylated) to 1 (completely methylated). We used the “noob” background normalization method46 to account for technical variation in back-ground signal fluctuations. The noob approach calculates on a natural logarithmic scale of the Infinium I design bead types to measure nonspecific fluorescence in the color channel opposite of their design (Cy3/Cy5).

DNAm age and epigenetic clock. DNAm levels give rise to particularly promising biomarkers of aging since chronological age (i.e., the calendar years that have passed since birth) has a profound effect on DNAm levels in most human tissues and cell types. To study the association of epigenetic AgeAccel with HD, we used 1 (Homozygote), 2 (Het), and 3 (Homozygote) for DNAm age on the respective datasets. DNAm age on the basis of 71 CpGs15, (3) DNAmAgeSkinClock on the basis of 391 CpGs16, (4) DNAm PhenoAge on the basis of 513 CpGs17, and (5) DNAmGrimAge on the basis of 1030 CpGs16. The first three DNAm ages were developed as a measure of chronological age, while the last two were developed as a measure of mortality risk. However, all the biomarkers are associated with mortality and morbidity18,47.

All of the DNAm age models were established through elastic net regression models. For example, the pan-tissue clock is based on methylation levels of 353 dinucleotide markers known as cytosine phosphate guanines or CGs15. The weighted average of these 353 epigenetic markers gives rise to an estimate of tissue age (in units of years), which is referred to as “DNAm age” or as “epigenetic age.” The pan-tissue epigenetic clock method applies to any tissue or cell type that contains DNA (with the exception of sperm), including individual cell types (helper T cells, neurons, glial cells) and complex tissues and organs (blood, brain, bone, kidney, liver, lung)44.

To study the association between the DNAm-based biomarkers and HD, we used a measure of epigenetic AgeAccel that has been widely used in our previous studies of aging. We regressed DNAm age on chronological age using a linear regression model and defined AgeAccel as the corresponding raw residuals. By definition, AgeAccel has no linear dependence on chronological age (correlation r = 0). Thus, a positive (or negative) value of AgeAccel indicates that the DNAm Age is higher (or lower) than expected based on age. In an analogous manner, we defined the acceleration measures, AgeAccelSkinClock, AgeAccelPheno, and AgeAccelGrim, based on DNAm AgeSkinClock, DNAm AgePheno, and DNAmGrim Age, respectively. We further examined two widely used acceleration measures: IEAA derived from Horvath’s pan-tissue DNAm Age, which is independent of age-related changes in blood cell composition, and extrinsic epigenetic age acceleration based on Hannum’s DNAm Age, which up-weights the contribution of blood cell count measure.

We adjusted the CpG data for sex using a linear model, then related each CpG to HD status using the robust biweight mid correlation. We used the “ebelmda” function in the GenABEL R package to calculate the inflation factors48.

We stratified our EWAS analysis by pre-manifest/manifest HD disease status because each stratum lends itself for addressing different biological questions: studying time to HD onset may involve different pathways from those that determine motor progression in manifest HD samples. Because sex can have an effect on DNAm levels, we adjusted the DNAm levels for sex by forming residuals. Since HD germline mutation status was not related to chronological age, there was no need to adjust for chronological age.

Our analysis methods make extensive use of meta-analysis. A simple yet powerful meta-analysis method, known as Stouffer’s method, relies on combining the Z-scores from individual datasets (the two blood datasets (aggregated Enroll-HD and the Registry-HD study). Specifically, for each CpG i and dataset a, one obtains a Z statistic Za,i, for example, by the inverse normal transformation of the p value. Next, a meta-analysis Za for each CpG is calculated as

\[ Z_a = \frac{1}{\sqrt{N_a}} \sum_{i} Z_{a,i} \]

The meta-analysis statistic Za is approximately normally distributed with mean 0 and variance 1; the corresponding p value is then calculated using the normal distribution.

Human brain methylation data in multiple regions. We studied the association of DNAm in methylated dinucleotide markers known as cytosine phosphate guanines or CpGs14. All of the DNAm age models were established through elastic net regression models. For example, the pan-tissue clock is based on methylation levels of 353 dinucleotide markers known as cytosine phosphate guanines or CGs15. The weighted average of these 353 epigenetic markers gives rise to an estimate of tissue age (in units of years), which is referred to as “DNAm age” or as “epigenetic age.” The pan-tissue epigenetic clock method applies to any tissue or cell type that contains DNA (with the exception of sperm), including individual cell types (helper T cells, neurons, glial cells) and complex tissues and organs (blood, brain, bone, kidney, liver, lung)14.

Finally, we re-examined model 5 (β = CAG.long × CAG.short), and (4) a model with three covariates CAG.long, CAG.short, and their interaction term. Polynomial regression models 5–8 evaluated the quadratic effect of CAG long. Models 5–7 extended models 1–3, respectively, by adding the square term of CAG long. Model 8 evaluated three covariates square of CAG long, CAG short, and their interaction term. Each model also included age at blood draw and sex. These models were evaluated in three subsets: all individuals, only controls, and only HD mutation carriers. We could not evaluate controls in the Registry-HD lymphoblastoid cells and the MTM fibroblast data due to low sample size (n = 25 controls).

CCG-repeat length versus HD methylation. We evaluated five multivariate linear regression models for studying the effect of CCG-repeat length on cg22982173 methylation (dependent variable) in the Registry-HD cohort (up to 372 observations). Model 1 involved two covariates: the two alleles measuring CCG-repeat length. Model 2 extended model 1 by adding two alleles measuring CCG-repeat length. Model 3 is the same as model 1, but limited to the analysis to 219 individuals with homozygosity for CGC (where z equals the CCG-repeat length). Model 4 involved both alleles for CCG length plus HD disease status. Finally, we re-examined model 5 (N = 350 observations) that included both alleles of CAG length, but omitted all HD cases that carried an atypical CAG CAG Repeat length. Model 8 evaluated the quadratic effect of CAG long. Models 5–7 extended models 1–3, respectively, by adding the square term of CAG long. Model 8 evaluated three covariates square of CAG long, CAG short, and their interaction term. Each model also included age at blood draw and sex. These models were evaluated in three subsets: all individuals, only controls, and only HD mutation carriers. We could not evaluate controls in the Registry-HD lymphoblastoid cells and the MTM fibroblast data due to low sample size (n = 25 controls).
repeats in the experimental cohort to avoid skewed distributions. Best Gaussian fit was judged by eye. Body weight cut off experimental animals had to weigh ≥11 g (females) and ≥14 g (males) by 5 weeks of age. Any presenting any anomaly were excluded. Unacceptable anomalies were cataracts, malocclusion, missing/ small eye, ear infection, unreadable, or missing tag. Mice were housed in cages enriched with two play tunnels, a plastic bone and enviro-dri® (Shepherd Specialty Papers). Animal cage changes occurred weekly. The cages were maintained on a 12:12 h light/dark cycle. Water and food were freely available at all times. This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals, NRC (2010). The protocols were approved by the Institutional Animal Care and Use Committee of Psychogenics, Inc., an AAALAC International accredited institution (Unit #001213).

The mouse methylation array study was conducted at UCLA. HD KI Q175 mice and wild-type littermate control mice were obtained from The Jackson Laboratory (JAX stock number: 370476). Animals were housed in standard mouse cages under conventional laboratory conditions, with constant temperature and humidity, 12 h/ 12 h light/dark cycle (7:00 a.m./7:00 p.m.) and food and water ad libitum. All animal studies were conducted in strict accordance with National Institutes of Health guidelines and approved by the UCLA Institutional Animal Care and Use Committees.

Mouse methylation data. For the custom methylation array study (Horvath-MammalMethylChip40), we profiled eight tissue samples for each tissue and group defined by disease status (Q175 and Q20). We used the Illumina array for five tissues: cerebellum, striatum, cortex, liver, and blood. In addition, we used another platform RRBS analysis to generated additional methylation data for the cerebellum and striatum (again eight samples per group and tissue). We limited the CpGs with at least 15 observations, yielding 2,896,456 and 2,965,343 CpGs available for the cerebellum and striatum EWAS, respectively. We performed Student's t tests for association tests and combined the results across the tissues based on the Stouffer's method.

RRBS is a widely used technology because it is cost-effective at measuring cytosine methylation data at hundreds of thousands (or even millions) of locations at a single-nucleotide level. Compared to whole-genome sequencing it is cost effective because it combines restriction enzymes and bisulfite sequencing to enrich for areas of the genome with a high CpG content. Its limitations include a bias towards regions with a high CpG content and relatively low coverage of individual CpGs (compared to an Illumina array).

The mammalian methylation array is attractive because it provides very high coverage (over thousand X) of highly conserved CpGs in mammals, but it focuses on only 37k CpGs that are highly conserved across mammals.

An ovine transgenic HD model. We analyzed a total of 168 sheep (57% females): 84 HD transgenic sheep age matched with 84 sheep as controls. The age of sheep ranged from 2.9 to 7.0 years with mean ± SD = 4.1 ± 0.8. HD transgenic sheep generated from a new large-animal HD transgenic ovine model. Sheep (Ovis aries L.) were selected because the developmental pattern of the ovine basal ganglia and cortex (the regions primarily affected in HD) is similar to the analogous regions of the human brain. A region of a full-length HDT DNA containing 73 polyglutamine repeats under the control of the human promoter resulted in six transgenic founders varying in copy number of the transgene.

To isolate genomic DNA, thawed blood samples (300 μL) were treated twice with red cell lysis buffer (300 μM sucrose, 5 mM MgCl2, 10 mM Tris pH 8, 1% Triton X (100), for 10 min on ice; each incubation was followed by centrifugation at 1800 rcf for 10 min at 4 °C, and removal of supernatant. Final pellet was incubated in cell digestion buffer (2.4 mM EDTA, 75 mM NaCl, 0.5% sodium dodecyl sulfate) and protease K (final concentration 500 μg/mL) for at least 2 h at 50 °C. An equal volume of phenolchloroform:isoamyl alcohol (25:24:1; pH 8) was added and samples mixed by inversion, followed by centrifugation at 14,000 rcf for 5 min at room temperature. This step was repeated if required. Supernatant was collected and mixed with 2× volume 100% ethanol to precipitate DNA. Following removal and evaporation of residual ethanol, genomic DNA samples were resuspended in 50 μL TE buffer (pH 8). Sample concentrations were initially measured using the NanoDrop spectrophotometer to further dilute samples to the desired 1000 μg for measurement on Qubit. All protocols were approved by the University of Auckland Animal Ethics Committee (New Zealand) and the SARDI/PIRSA (South Australian Research and Development Institute/Primary Industries and Regions South Australia) Animal Ethics Committee (Approval number 19/02).

Moreover, all work involving OVT73 Sheep was approved by PIRSA Animal Ethics Committee with oversite from the University of Auckland Animal Ethics Committee. DNAm arrays were profiled using a custom Illumina methylation array (HorvathMammalMethyl40) based on 38,000 CpG sites in highly conserved regions in mammals. EWAS was performed using the R function “standardScreeningNumericTrait” from the “WGCNA” R package.

HD motor progression in Enroll-HD data 1. To establish a measure of HD motor progression, we used the large-scale Enroll-HD database comprising 14,850 longitudinal observations across 5204 manifest HD individuals. Of the 5204 manifest individuals in the Enroll-HD data 2, the data 2 aimed to profile two longitudinal measures of DNAm array with approximately a 7-year gap; thus, most of the first blood samples were collected during visits by the individuals in the Randomized OVT73 HD study. We removed individuals that lacked Enroll-HD data and the visits with motor scores <5, leaving 275 manifest individuals (including 73 converting to manifest) remaining in our progression analysis. In establishing the measure of HD progression, there were >1860 observations across different visits available for each individual, with a mean follow-up period of 7.9 years, from the visits aligned with the first DNAm methylation profiles through to the most recent visits. The model framework of the linear mixed analysis was similar to the one used for Enroll-HD data 1, whereas the age at fixed effect was based on the visit aligned with the first methylation profiles. The random slopes with respect to visits were also adjusted for sex, age, CAG-repeat length, age at motor onset, and education attainment, and used the residuals as the measure of the progression for downstream analysis.

Assessment of HD progression in HTT cg22982173. We studied the implication of HTT cg22982173 in HD progression across different clinical assessment domains. While our primary measure of HD progression was based on motor scores assessments across several visits following the blood draw, we also defined analogous measures of progression across other clinical assessments: functional assessment score, the Stroop color and word test.

Detailed visualization of regional EWAS coMET. We applied the R package comet (version 3.1) to visualize the genomic regions of interest from our EWAS results.

EWAS of HD motor progression. We conducted the EWAS of motor progression scores based on the adjusted random slopes on Enroll-HD data 1 and data 2, respectively, and based on the adjusted last motor scores on the Registry-HD data. EWAS was performed with the function “standardScreeningNumericTrait” in the WGCNA R package under R 3.4.3. Effect sizes were based on bivariate mid-correlation (bicor) for robust correlation estimates. We combined the results across the three studies via fixed-effect models weighted by inverse variance under the random-effect model (released 2011–03–25) and only used the overlap CpGs between the Illumina 450k and EPIC array.

Gaphunter analysis to detect confounding by SNPs. The Gaphunter software allows one to identify CpG probes that are confounded by adjacent SNPs. We applied minP/Gaphunter (3.6) under R to our blood DNAm data from Enroll-HD data 1, Enroll-HD data 2, and Registry-HD, respectively. The analysis was performed using the R minP function, which identifies CpG probes with a gap in a (methylation) beta signal greater than or equal to the defined threshold and reports number of clusters constituting the gap. We used the default threshold of 0.05 and a default cut off value of 0.01. Evidence for SNP confounding exists if the CpG exhibits at least two distinct clusters that involve >20 individuals each. Gap patterns were examined in the 33 CpGs associated with HD disease status and the three CpGs associated with motor progression manifest HD cases.

Functional enrichment analysis. The AnRichment software is implemented in the online tool HDinHD29 (www.HDinHD.org), which includes published gene sets,
including GO (gene ontology) terms\textsuperscript{53}, KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways\textsuperscript{54}, Molecular Signatures Database gene sets\textsuperscript{55}, and curated literature gene sets included in the “userListEnrichment” function\textsuperscript{56} of the WGCNA (3.6.3) R package as well as transcriptomic co-expression network modules identified in analyses of publicly available expression data from HD patients as well as mouse models. The GO/METH enrichment analysis properly takes into account the different number of probes per gene present on Illumina array (450k in our analysis) by using Wallenius’ noncentral hypergeometric test\textsuperscript{50}. We used the GO/METH R function (R version 3.6.3), which includes GO terms and KEGG pathways.

**Reporting summary.** Further information on research design is available in the Nature Research Reporting Summary linked to this article.

**Data availability**

Our data are available from two data repositories. First, Gene Expression Omnibus (Supereeries GSE147004 and suberies GSE146917, GSE147002, GSE147003, and GSE27778 (human brain methylation). Second, from Enroll-HD [https://www.enroll-hd.org/]. Please direct inquiries to info@chdilfoundation.org with the words “Enroll-HD Methylation data” in the subject line. All other relevant data supporting the key findings of this study are available within the article and its Supplementary information files or from the corresponding author upon reasonable request. A reporting summary for this Article is available as a Supplementary information file.

**Code availability**

This article does not use any custom code or mathematical algorithm. Relevant code or software has been published in previous articles (“Methods”). In addition, software code is available upon request.

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Author contributions

S.H., X.W.Y., R.G.S., D.M., and A.T.L. conceived of the study. A.T.L. carried out most of the statistical analyses. P.N., M.J.G., C.S.B., S.R.R., T.A.H., and R.G.S. conceived and carried out the experiments of ovine transgenic Huntington’s disease model. N.W. and X.W.Y. conceived and carried out the experiments of Htt Q175 mouse model. P.L. carried out gene enrichment analysis and system biology-related analysis. G.C. helped brain-based methylation data analysis, M.C., A.C., A.M., and D.M. designed and carried out the implications of glutamine-encoding DNA sequence structures in HD methylation patterns. S.K., H.W., R.C., J.C., J.R., A.P., and T.F.V. contributed data, helped with the write up, and participated in the interpretation of the results. All authors participated in the interpretation of the study and helped revise the article.

Competing interests

UC Regents (the employer of S.H. and A.T.L.) has filed patents surrounding several epigenetic biomarkers of aging (including GrimAge), which list S.H. and A.T.L. as inventors.

Additional information

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