

BRAIN COMMUNICATIONS

Longitudinal expression changes are weak correlates of disease progression in Huntington's disease

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Huntington's disease is a severe but slowly progressive hereditary illness for which only symptomatic treatments are presently available. Clinical measures of disease progression are somewhat subjective and may require years to detect significant change. There is a clear need to identify more sensitive, objective and consistent measures to detect disease progression in Huntington's disease clinical trials. Whereas Huntington's disease demonstrates a robust and consistent gene expression signature in the brain, previous studies of blood cell RNAs have lacked concordance with clinical disease stage. Here we utilized longitudinally collected samples from a well-characterized cohort of control, Huntington's disease-at-risk and Huntington's disease subjects to evaluate the possible correlation of gene expression and disease status within individuals. We interrogated these data in both cross-sectional and longitudinal analyses. A number of changes in gene expression showed consistency within this study and as compared to previous reports in the literature. The magnitude of the mean disease effect over 2 years' time was small, however, and did not track closely with motor symptom progression over the same time period. We therefore conclude that while blood-derived gene expression indicators can be of value in understanding Huntington's disease pathogenesis, they are insufficiently sensitive to be of use as state biomarkers.

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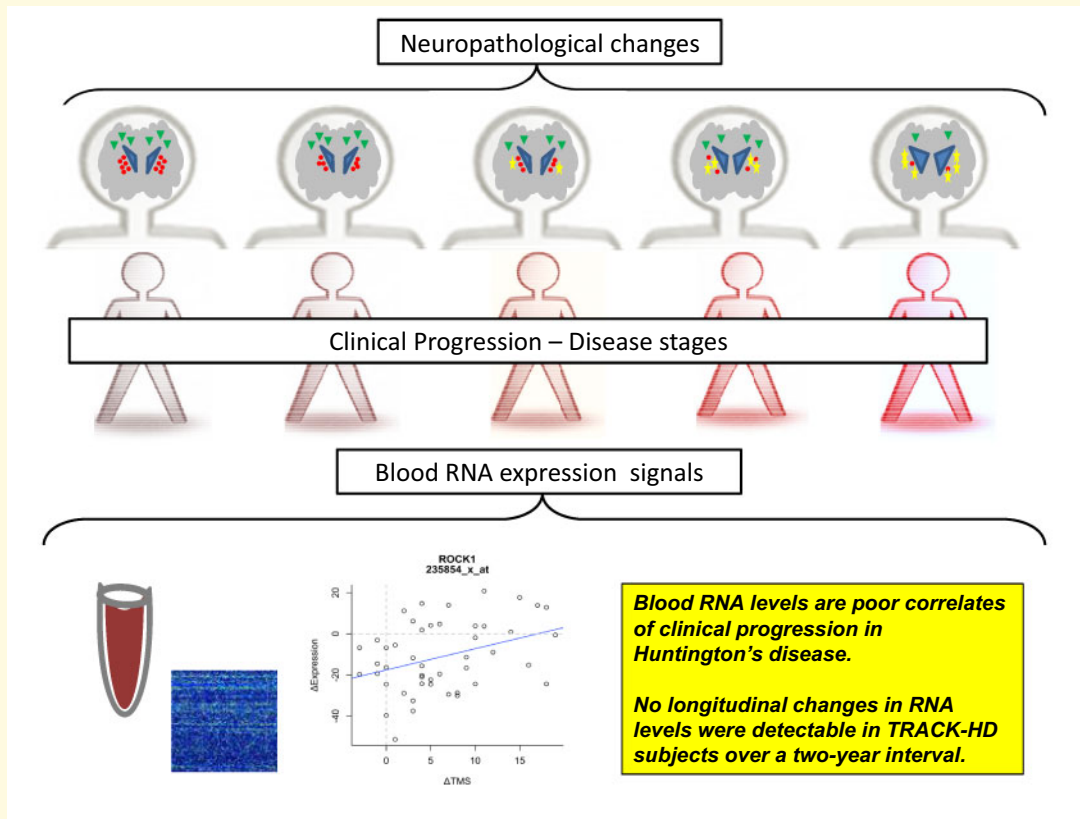
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Abbreviations: EST = Expressed Sequence Tag; FDR = false discovery rate; GSEA = gene set enrichment analysis; HTT = huntingtin; mHTT = mutant HTT; TMS = Total Motor Score; UHDRS = Unified Huntington's Disease Rating Scale.

Graphical Abstract



Introduction

Huntington's disease is a chronic, incurable and ultimately fatal neurodegenerative disease affecting an estimated 12 individuals per 100 000 in the UK (Evans *et al.*, 2013), and ~6 per 100 000 in the USA, Europe and Australia (Pringsheim *et al.*, 2012). It is caused by an expanded CAG trinucleotide repeat within the *huntingtin* (*HTT*) gene inherited in an autosomal dominant manner (Huntington's Disease Collaborative Research Group, 1993). This gives rise to an extension of a polyglutamine tract near the N-terminus of the HTT protein, which is expressed ubiquitously throughout the body. Huntington's disease pathogenesis is complex, involving multiple cellular mechanisms including protein aggregation, mitochondrial dysfunction and transcriptional dysregulation (Luthi-Carter, 2007).

Mutant HTT (mHTT) has a progressive and ravaging effect on the central nervous system, to the greatest extent in the striatum (Ross and Tabrizi, 2011). Cell death and dysfunction in Huntington's disease brain regions

give rise to a range of motor signs and symptoms, including chorea, alongside cognitive, behavioural and psychiatric disturbances (Walker, 2007; Stout *et al.*, 2011). Current treatments rely on symptomatic relief, although several promising experimental treatments are being developed that show potential to slow the disease (Dickey and La Spada, 2018).

Having an established trait marker, the extended CAG repeat, makes Huntington's disease unusual among neurodegenerative disorders. Presymptomatic predictive genetic testing can confirm whether an individual is carrying this mutation, providing an opportunity to study Huntington's disease development before onset and the possibility of presymptomatic treatment (Paulsen *et al.*, 2008). However, measuring presymptomatic Huntington's disease progression is difficult; insidious non-motor changes often precede more apparent extrapyramidal signs, although these effects can occur in either order (Tippett *et al.*, 2017).

Estimating the prospective age of disease onset from the length of the individual's CAG repeat can serve as a

crude proxy for disease state before diagnosis (Langbehn *et al.*, 2004) as age of onset is inversely correlated with the length of CAG expansion [with mitigating effects of interruption by a CAA codon (Wright *et al.*, 2019)]. This approach nonetheless has very limited precision, since the CAG repeat length only accounts for a maximum of 60% of variation in disease onset (Wexler *et al.*, 2004), and has an even lower effect in individuals carrying disease alleles with fewer than 50 repeats, which comprise the majority of Huntington's disease patients. Remaining heritable variation in age of onset may be accounted for in part by mutations in DNA maintenance genes (Genetic Modifiers of Huntington's Disease Consortium, 2019).

In manifest Huntington's disease, measurement of disease progression is also complicated by extensive variation of the clinical phenotype and the slow rate of progression. The Unified Huntington's Disease Rating Scale (UHDRS), a combined clinical assessment of motor function, functional capability, cognition and behaviour (Huntington Study Group, 1996) is the predominant method of tracking disease severity over time. Observational scales are subjective, however, and therefore prone to bias and inter-rater variability (Henry *et al.*, 2005). More sensitive, robust and objective methods of tracking Huntington's disease progression are therefore urgently needed to make the most of longitudinal clinical studies and especially to assess the potential benefits of new therapies in clinical trials.

Several different approaches have been taken to develop Huntington's disease biomarkers that can monitor Huntington's disease progression, both before and after disease manifestation. For these to be useful they should be highly reproducible, show little variability, and show quantitative, sensitive association with disease progression (Weir *et al.*, 2011). Such biomarkers would considerably reduce the number of participants needed for clinical trials and aid the identification of effective treatments (Paulsen *et al.*, 2006). Furthermore, the search for biomarkers could give further insight into the pathology of Huntington's disease and potentially reveal novel targets for treatment.

Transcriptional dysregulation is a hallmark of Huntington's disease, thereby making RNA measurements an attractive area for potential biomarker development. Over almost two decades, genome-wide expression techniques have built a comprehensive picture of the transcriptional alterations mHTT provokes in the brain through multiple cellular mechanisms (reviewed in Seredenina and Luthi-Carter, 2012). Extensive changes in mRNA expression across human Huntington's disease brain regions has been associated with vulnerability to degeneration, establishing close links between transcriptional dysregulation and Huntington's disease pathology (Hodges *et al.*, 2006). Several studies have identified parallel changes in Huntington's disease mouse models (Luthi-Carter *et al.*, 2000; Kuhn *et al.*, 2007) which show progression over

time and correlation with losses in motor function and behavioural disturbances (Luthi-Carter *et al.*, 2002a; Kuhn *et al.*, 2007; Giles *et al.*, 2012). Not all expression changes contribute to the disease process; some changes appear to have no pathological effect (Rudinskiy *et al.*, 2009), while others are compensatory (Seredenina *et al.*, 2011).

The transcriptional regulatory pathways known to be altered by mHTT are involved in gene expression in multiple tissues throughout the body in model systems (Sugars and Rubinsztein, 2003). Parallel gene expression changes have been observed in R6/2 mouse and human Huntington's disease muscle (Luthi-Carter, *et al.*, 2002b; Strand *et al.*, 2005) and correlate with muscle atrophy. Expression changes have also been observed in Huntington's disease mouse liver and pancreas (Andreassen *et al.*, 2002; Chiang *et al.*, 2011), and brown and white adipose tissues (Weydt *et al.*, 2006; Phan *et al.*, 2009), including genes related to energy expenditure (McCourt *et al.*, 2016) that could contribute to weight loss. Data from model systems provide evidence that mHTT accumulation in peripheral tissues may cause functional abnormalities independently of neurodegeneration, including muscle strength, osteoporosis, cardiac failure and impaired glucose tolerance (van der Burg *et al.*, 2009). Transcriptional changes have also been described in human Huntington's disease fibroblasts (Marchina *et al.*, 2014).

Peripheral blood, being a renewable, easy-to-collect tissue, is an attractive substrate for biomarker development. Several exploratory studies have reported transcriptomic changes in Huntington's disease blood and highlighted specific RNAs as potential biomarkers. These have used both genome-wide (microarray, DeepSAGE) and targeted reverse transcription real-time quantitative PCR gene expression analyses (Borovecki *et al.*, 2005; Runne *et al.*, 2007; Lovrecic *et al.*, 2010; Hu *et al.*, 2011; Cesca *et al.*, 2015; Mastrokolas *et al.*, 2015). Despite the number of potential biomarkers identified, there has been little overlap between the reported signatures across these studies. Results of focused attempts to cross-validate results have also been largely negative (Runne *et al.*, 2007; Lovrecic *et al.*, 2009; Mastrokolas *et al.*, 2015; Zadel *et al.*, 2018).

Additional studies have taken a somewhat different approach by considering transcriptional changes within the contexts of identified etiologic processes in Huntington's disease (Krzysztoń-Russjan *et al.*, 2013; Träger *et al.*, 2014; Miller *et al.*, 2016). If differential expression of genes identified by these studies can be replicated, they may also warrant consideration as potential biomarkers. Lack of concordance across these studies has hindered the implementation of RNA-based Huntington's disease biomarkers. Discrepancies have been attributed to technological limitations, methodological inconsistencies and human blood sample variability (Rodrigues *et al.*, 2018).

The development of blood-based state biomarkers for Huntington's disease has also been limited by the lack of longitudinal studies. On the positive side, sequential analyses of samples from individual subjects might minimize effects of patient-to-patient variability (Tabrizi *et al.*, 2009). On the other hand, cross-sectional studies may maximize detection of RNA changes in Huntington's disease versus control subjects because they reflect a long and cumulative process of mHTT exposure (Weir *et al.*, 2011). To be useful, however, longitudinal measures need to detect changes that occur in a timeframe relevant for clinical trials, e.g. 1–3 years.

It has also been suggested that independence of neurological and peripheral Huntington's disease pathologies might fundamentally limit the utility of using transcriptional changes in the blood to track the progression of Huntington's disease (Zadel *et al.*, 2018). However, previous claims of parallels between transcriptional dysregulation in the blood and brain have challenged this notion (Mastrokolas *et al.*, 2015; Mina *et al.*, 2016). One study purported to show similarities between Huntington's disease blood and brain expression signatures through gene set enrichment analysis (GSEA) (Hensman Moss *et al.*, 2017). However, none of the individual genes within these sets was significantly differentially expressed in Huntington's disease compared to controls after appropriate false discovery rate (FDR) correction, raising caveats in these conclusions. Questions therefore remain over the nature and degree of similarities between neurological and peripheral transcriptional dysregulation, and whether these changes progress at rates that allow blood mRNA to be used as a reliable predictor of neurological disease progression.

This study aimed to evaluate potential mRNA state biomarkers for Huntington's disease in human blood samples collected in the TRACK-HD study (Tabrizi *et al.*, 2009). TRACK-HD was a large multi-site observational study in which quantitative MRI, cognitive, motor and neuropsychiatric assessments were collected in more than 350 subjects, comprising individuals with an Huntington's disease diagnosis, individuals at risk for Huntington's disease (carrying an expanded *HTT* allele) and control subjects. The high standardization of these measures across sites and the relatively long 24-month follow up time with individual subjects provided an opportunity to compare established clinical indicators with additional phenotypic measures. We therefore undertook transcriptomic analyses in this well-characterized subject population.

We performed both cross-sectional and longitudinal microarray gene expression analyses of TRACK-HD blood samples from Huntington's disease subjects in early clinical stages of disease, presymptomatic carriers of the Huntington's disease mutation, and controls, collected from the same set of individuals 2 years apart (see also Cohort below). These data were analysed cross-sectionally at both timepoints and also used to construct a

longitudinal analysis of gene expression and disease progression. Both of these analyses were then interrogated to identify novel candidate biomarker genes, and to test progressive differential expression of genes identified in previous studies, using publicly available datasets (Borovecki *et al.*, 2005; Runne *et al.*, 2007; Lovrecic *et al.*, 2010; Hu *et al.*, 2011; Krzysztoń-Russjan *et al.*, 2013; Träger *et al.*, 2014; Cesca *et al.*, 2015; Mastrokolas *et al.*, 2015; Miller *et al.*, 2016; Zadel *et al.*, 2018), as well as an independent TRACK-HD sample cohort. Finally, we sought to shed new light on, or to reproduce previous evidence of, molecular mechanisms of Huntington's disease pathogenesis identified using GSEA (Hensman Moss *et al.*, 2017). We confirm that blood RNA changes may reflect small Huntington's disease-related effects, but find that such measures are likely to be unsatisfactory as clinical state biomarkers of Huntington's disease.

Materials and methods

Study cohort

Whole blood samples were collected from participants in the Track-HD in London and Vancouver (Supplementary Tables 1 and 2) into PAXgene Blood RNA tubes (Qiagen). Presymptomatic subjects [UHDRS Total Motor Score (TMS) ≤ 5] were further stratified based on their estimated time to diagnosis based on their current age and their estimated age of onset (calculated using the repeat length of their expanded *HTT* allele) according to (Langbehn *et al.*, 2004). Subjects were then classified as farther (>10.8 years) from predicted onset (preHD A) or nearer (<10.8 years) to predicted onset (preHD B) (Supplementary Table 3). Diagnosed early symptomatic Huntington's disease participants were classified by the Total Functional Capacity score of the UHDRS into Stage 1 (zHD1, Total Functional Capacity = 11–13) and Stage 2 (zHD2, Total Functional Capacity = 7–10) disease subgroups (Tabrizi *et al.*, 2009, 2012). The subset of samples represented balanced numbers of preHD and HD cases at each stage. The study was approved by the local ethics committees and written informed consent was obtained from each subject according to the Declaration of Helsinki.

Microarray analyses

DNA microarray analyses were conducted using Affymetrix Human Genome U133 Plus 2.0 Arrays. Expression values were normalized using the RMA algorithm included in the Limma package (Bioconductor). A linear regression model was fitted and adjusted to remove the effects of sample origin (London or Vancouver), gender and age. *P*-values were adjusted using the FDR method (Benjamini and Hochberg, 1995).

Cross-sectional comparisons of microarray gene expression in Huntington's disease versus control samples at each time-point were performed using R. Cross-sectional microarray analyses of differential expression were performed in Year 1 samples of 37 diagnosed Huntington's disease subjects (18 zHD1, 19 zHD2) versus 24 controls. Year 3 samples were tested in the same manner and included 32 diagnosed HD subjects (16 zHD1, 16 zHD2) and 20 controls. The Year 3 samples were collected from a subset of participants whose samples had been analysed in Year 1. We also conducted cross-sectional analyses including all presymptomatic samples (data not shown), but did not draw conclusions from these analyses, as no differential expression could be detected.

Analyses of gene expression versus disease state

Cohorts for longitudinal analyses comparing microarray expression and TMS were selected by comparing mean TMSs of sample groups to the control group using Dunnett's multiple comparisons test of an ordinary one-way ANOVA, performed using Prism 7 (GraphPad; [Supplementary Table 3](#)). On this basis, we excluded preHD A samples, as the median TMSs of these groups were not significantly different from that of the control group.

Longitudinal analyses included samples from 15 presymptomatic Huntington's disease gene carriers (all preHD B) and 32 diagnosed Huntington's disease subjects (16 zHD1, 16 zHD2). An additional longitudinal analysis of gene expression was performed in samples from a control cohort of 20 individuals for sake of comparison; as expected, this group of individuals did not show a change in TMS over the 2-year period.

Microarray gene expression data was analysed and normalized using R, as described above. Expression values were unlogged and the change in gene expression (Δ Expression) was calculated for each individual across 2 years. Change in TMS (Δ TMS) was plotted against Δ Expression and fitted with a linear regression model. Pearson's correlation coefficient was calculated and the *P*-values were also adjusted using FDR. A cross-sectional analysis of relationship between gene expression and TMS was performed at each individual time-point (Year 1, Year 3). A longitudinal gene expression analysis was also performed on the control samples (without consideration of Δ TMS).

Pathway analyses

GSEA (Broad Institute; [Subramanian et al., 2005](#)) was performed on expression data of 97 Year 1 samples, implementing the analysis parameters of [Hensman Moss et al. \(2017\)](#). This included a control group of 24 samples and a Huntington's disease group of 73 samples (including all four presymptomatic and symptomatic

Huntington's disease groups). Genes were ranked by *t*-test of differential expression between the Huntington's disease and control groups. We then tested 5519 overlapping pathway gene sets obtained from the Gene Ontology, REACTOME, Kyoto Encyclopedia of Genes and Genomes, NCI Pathway Interaction Database and BioCarta databases, focusing testing on sets containing between 3 and 500 genes. The GSEA method calculated enrichment scores for each set reflecting representation of that pathway at the top and bottom of the ranked gene list (i.e. the most probably differentially expressed genes in Huntington's disease, both increased and decreased). To estimate the statistical significance of the GSEA enrichment scores obtained, we used a sample label permutation strategy to estimate the null distribution (as recommended by [Mooney and Wilmot, 2015](#)). This involved permuting the phenotype labels 1000 times and generating enrichment scores and *P*-values in the same manner. Finally, *q*-values were calculated from the *P*-value distributions. *q*-Values were calculated separately for up- and downregulated gene sets.

Data availability

The NCBI Gene Expression Omnibus accession number for the microarray data reported in this paper is GSE135589.

Results

Microarray differential expression analyses in Huntington's disease versus control subjects from the TRACK-HD study

The Track-HD study provided an opportunity to obtain a considerable number of samples collected in a highly standardized manner from a clinically well-characterized patient cohort ([Tabrizi et al., 2009](#)). We reasoned that analyses of TRACK-HD samples therefore maximized the chances of discovering or validating blood RNA biomarkers for Huntington's disease progression. To this end, microarray gene expression analyses were conducted from Year 1 total blood RNA samples from 37 manifest Huntington's disease subjects, 35 presymptomatic Huntington's disease gene carriers and 24 controls ([Tabrizi et al., 2009](#)) (see also Materials and Methods and [Supplementary Tables 1 and 2](#) for further details). As most of these subjects would carry on participating in the study to its completion, we also had the opportunity to perform longitudinal analyses of Year 3 samples (see below).

To highlight the largest potential disease-related changes, we first conducted analyses of samples from symptomatic Huntington's disease subjects versus

controls. For the Year 1 microarray data, these comprised samples from 37 Huntington's disease subjects and 24 controls, while Year 3 data comprised 32 of Huntington's disease subjects and 20 controls. A linear regression model was fitted to analyse the relationship between expression and condition. The model also included gender, age and sample collection site (London or Vancouver) to avoid confounding effects.

Analyses of Year 1 samples detected 2575 probesets of 54 675 that reported differential expression at cutoff of nominal $P < 0.05$ between the control and Huntington's disease groups [1125 increased and 1450 decreased in Huntington's disease (Fig. 1 and Supplementary Table 4)]. Supplementary Table 4 also lists the 20 probesets with most significant differential expression and the functions of proteins encoded by the genes they represent, which include gene expression (*RORC*, *ZFP42*) and involvement in cell structure (*IQCA1*, *DYNCL12*). This number of changes does not convincingly exceed chance levels, however. Also consistent with the null hypothesis, multiple testing correction by a well-established method assigned all FDR P -values (q -values) to be greater than 0.05.

Because most participants remained in the study for its duration, analyses of RNA samples from most of the

same subjects were analysed again after a 2-year interval (TRACK-HD study Year 3). In the Year 3 analysis, 3261 probesets showed a potentially significant change of expression between control and Huntington's disease subjects [by the criterion of nominal $P < 0.05$; 1962 upregulated, 1299 downregulated (Fig. 1 and Supplementary Table 5)], but still no individual probesets showed differential expression that could be considered significant after correction for multiple testing.

The 20 probesets with the lowest P -values in comparisons between Huntington's disease and control samples are also listed in Supplementary Table 5; these include probes for genes whose products are involved in gene regulation (*FOXK2*, *MRGBP*), are active as serine/threonine kinases (*SGK494*, *AKT3*, *ATM*) and comprise another glutamine-rich protein (*QRICH1*). Despite the apparent low level of differential expression signal, there was a small overlap of differential gene expression signatures between the two time-points (Table 1). Of the probesets meeting criteria of nominal $P < 0.05$ in the Year 1 data, 299 meet the same criteria for differential expression in Year 3, with 270 of these the changing in the same direction. The high ratio of concordant versus discordant changes across two separate experimental analyses suggests that these positive results are not due to technical variability.

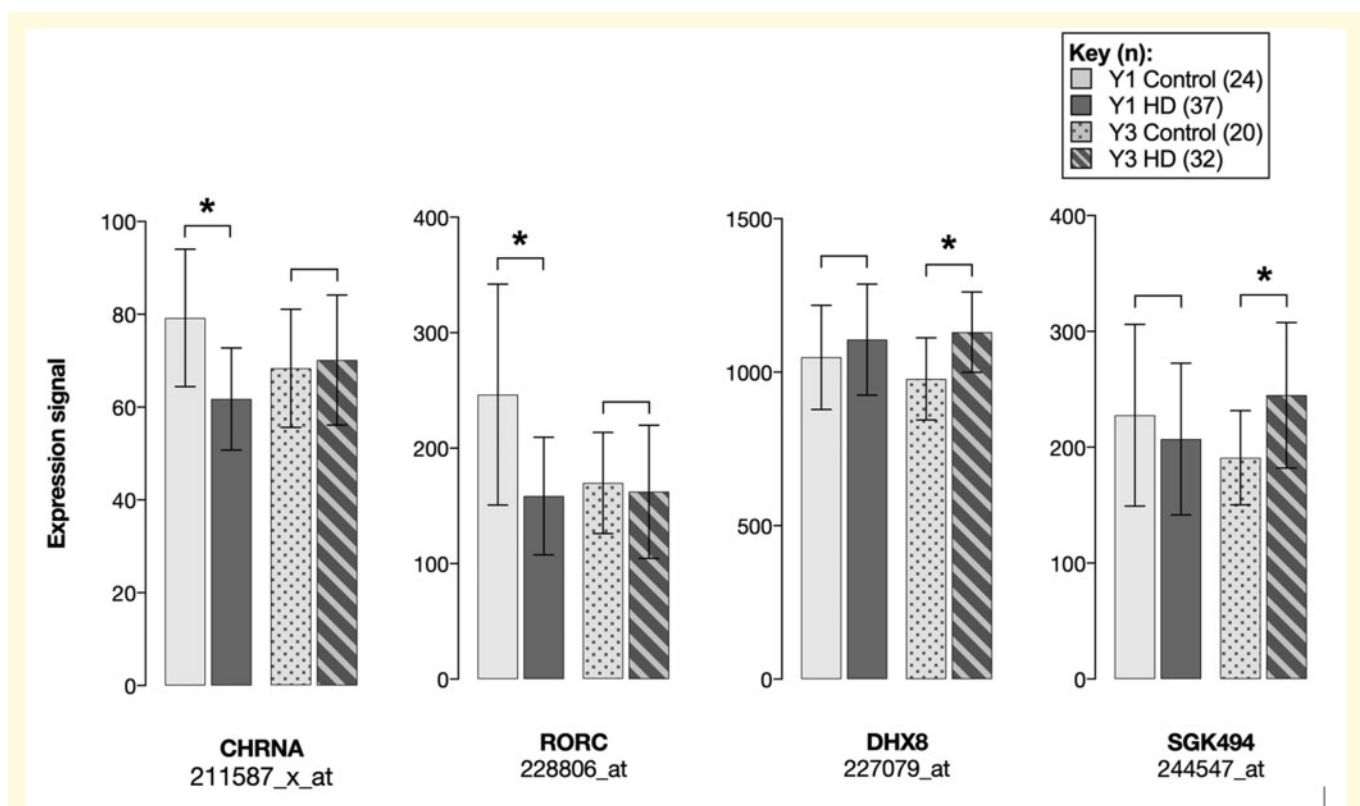


Figure 1 Gene expression of four probesets that showed the strongest evidence of differential expression in Huntington's disease subjects compared to controls. Asterisks indicate nominal $P < 0.05$. Adjusted P -values > 0.05 for all genes. Error bars represent SEM. Expression signals are unlogged for display purposes.

Table 1 Top 5 most significant probesets from each year showing significant differential expression at the other time point

| Gene symbol | Probeset | Protein function | Year 1 | | Year 3 | |
|-------------|-------------|---|--------|---------|--------|---------|
| | | | logFC | P | logFC | P |
| HSA277841 | 1560855_at | ELG protein | -0.34 | 1.5E-04 | 0.24 | 0.038 |
| MARCH8 | 231933_at | Ubiquitin ligase | -0.57 | 8.7E-04 | -0.36 | 0.046 |
| TRIM58 | 215047_at | Ubiquitin ligase | -0.59 | 8.8E-04 | -0.44 | 0.013 |
| TOMM34 | 201870_at | Transport across mitochondrial membrane | 0.23 | 9.1E-04 | 0.25 | 5.5E-03 |
| LIMS1 | 207198_s_at | LIM and senescent cell antigen-like domains | -0.25 | 1.3E-03 | -0.18 | 0.027 |
| SESN3 | 235683_at | TORC1, TORC2 signalling pathways | -0.41 | 0.042 | -0.68 | 2.7E-04 |
| ELL2 | 214446_at | Elongation factor for RNA polymerase II | -0.44 | 8.6E-03 | -0.55 | 3.0E-04 |
| CYP19A1 | 239459_s_at | Cytochrome P450 family member | -0.27 | 0.038 | -0.58 | 7.3E-04 |
| CCDC15 | 220466_at | (Unknown) | -0.33 | 0.046 | -0.49 | 7.3E-04 |
| N4BP2L2 | 214753_at | (Unknown, associated with immunity) | 0.19 | 3.7E-03 | 0.20 | 7.4E-04 |

P-values have not been adjusted for multiple testing. EST. Protein function is inferred from GeneCards Human Gene Database.

Focused analyses of blood RNA differential expression measures identified by previous Huntington's disease studies

A disease state biomarker should ideally be universally applicable, such that it is detectable in Huntington's disease versus control samples in different laboratories and independent sample cohorts. Replication of potential blood RNA biomarker signatures in Huntington's disease has been generally inconsistent in this regard. We nonetheless examined whether RNA changes identified as potential biomarkers in previous studies were represented in our analyses of differential expression between Huntington's disease subjects and controls (Supplementary Table 6). This included a panel of 12 potential biomarker genes identified through microarray analysis of whole blood by (Borovecki *et al.*, 2005) and 2 further genes discovered through machine learning analysis of the same microarray data (Lovrecic *et al.*, 2010). It also examined the expression of the histone gene *H2AFY* identified by (Hu *et al.*, 2011) and subsequently analysed in (Mastrokolias *et al.*, 2015). Five additional differentially expressed blood RNAs identified by (Mastrokolias *et al.*, 2015) using DeepSAGE were also evaluated. Finally, we tested seven other RNAs showing differential expression by microarray analyses of Huntington's disease and control blood samples published recently by (Zadel *et al.*, 2018).

We also considered the results of five additional RNA studies that focused on putative mechanisms of disease. One RNA was included from a previous study of inflammatory processes in lymphocytes (Runne *et al.*, 2007). Two further candidates were chosen from a study that considered Ca^{2+} homeostasis and neuroprotection using mononuclear blood cells (Cesca *et al.*, 2015). Six genes were identified based on a study of metabolic- and cellular stress-related RNA measures in whole blood compared to other clinical, anthropometric and biochemical parameters (Krzysztoń-Russjan *et al.*, 2013). Larger gene

sets were also considered based on data from Huntington's disease monocytes: these comprised the 20 most significantly dysregulated genes from a quantitative PCR study of the NF κ B pathway (Träger *et al.*, 2014) and the top 20 from a full RNA-Seq analysis (Miller *et al.*, 2016).

All of these candidate genes had been previously subject to technical validation using quantitative PCR in their respective initial reports. The majority of genes from earlier studies had been selected for independent validation in additional cohorts, with limited success. To the best of our knowledge this study is the first to attempt to reproduce the positive biomarker study results of Cesca *et al.* (2015) and Krzysztoń-Russjan *et al.* (2013).

In total, 70 unique transcripts identified in previous studies were tested in our two cross-sectional differential expression analyses. In the Year 1 Huntington's disease versus control analysis, 8 of these showed statistically significant differential expression ($P < 0.05$, without FDR correction). However, only two of these transcripts (*SAP30* and *ENSA*) were dysregulated in the same direction as reported in their original studies. The protein encoded by *SAP30* is a subunit of the histone deacetylase complex, noteworthy due to the well-established interference by mHTT of the regulatory machinery of gene expression. *ENSA*, which has been suggested as a candidate gene for insulin dependent diabetes mellitus (Héron *et al.*, 1999), encodes the endogenous ligand of the ABCC8 regulatory subunit of K_{ATP} channels. The Year 3 analysis showed even less concordance: 6 genes were significantly dysregulated, yet only *LTBR* (which encodes the lymphotoxin- β receptor) showed a change in the same direction as previously reported. The candidate biomarker panel identified by Krzysztoń-Russjan *et al.* (2013) showed a high proportion of statistically significant transcripts in both years; however all of these were dysregulated in the opposite direction to that which was originally reported, which suggests that these are genes that show a large degree of heterogeneity of expression (or detection) between individuals, independent of Huntington's disease status.

Global longitudinal analysis of gene expression versus TMS

It is essential for a disease state biomarker to show a clear relationship to disease progression. A robust way of assessing this is to identify and evaluate potential biomarkers is to follow this potential relationship in a single cohort longitudinally. This also provides a means of testing biomarkers that largely circumvents the interference of inter-individual variability.

Along with blood RNA microarray data, the Track-HD study collected clinical measurements including TMS, the component of UHDRS that assesses 31 motor symptoms of Huntington's disease; the higher the score, the more severe the disease state (Huntington Study Group, 1996). To focus our analysis on individuals with significantly progressive disease, the cohort for the longitudinal analysis was selected through analysis of the mean TMS change across 2 years of each sample group (Supplementary Table 3). As such, the analysis included 15 presymptomatic *HTT* mutation carriers and 32 diagnosed Huntington's disease subjects. In this analysis, Δ TMS was used as a scale of disease progression across the 2 years and was plotted against Δ Expression in each individual. For each microarray probeset, a linear regression analysis was performed to model the slope and statistical significance of the relationship between the two variables (Fig. 2). Pearson's correlation coefficient was used to determine the degree to which they were related.

The 20 probesets that showed most significant correlation are displayed in Table 2; 4273 probesets, out of a total of 54 675, showed a statistically significant correlation ($P < 0.05$) (Supplementary Table 7); 2962 of these showed positive correlation and 1311 an inverse relationship. Furthermore, 394 probesets showed very highly significant correlation ($P < 0.005$). Notably, however, when multiple testing correction was applied, the significance of association for all probesets was $P > 0.05$. The most significant 20 probesets included genes that encoded proteins involved in mitochondrial maintenance (*HSPA9*), transcriptional regulation (*NACCI*) and cell signalling pathways (*LYPD6*).

To further assess the genes identified for their ability to detect Huntington's disease progression, a comparative longitudinal analysis was performed in a group of 20 controls from the Track-HD study. It would be expected that expression changes associated with Huntington's disease progression would not be seen in a control group. However, in this analysis, 5392 probesets showed very highly statistically significant (adj. $P < 0.005$) differential expression. These included 319 of the 4273 statistically significant probesets identified in the Huntington's disease correlation analysis. This result suggests that some of the signal observed might be due to technical variation (e.g. due to microarray batch processing).

The utility of a potential biomarker would be increased if it displayed a clear association with disease state at

cross-sectional time-points, to inform clinical trial recruitment, for example. Therefore, cross-sectional analyses of the relationship between TMS and expression were also conducted. These included the sample groups with a significantly different mean TMS than the control group (at both timepoints) (Supplementary Table 3). In the Year 1 samples, 3991 probesets showed statistically significant ($P < 0.05$) association with TMS, including 467 of the changes identified in the longitudinal correlation analysis (Supplementary Table 8). At Year 3, the number of probesets with a statistically significant ($P < 0.05$) relationship between TMS and expression was 1601. Of these, 255 probesets had been also identified as significant in our longitudinal profiling (Supplementary Table 9).

Evaluation of gene signatures from previous studies in our longitudinal analysis

Along with a lack of replication of potential Huntington's disease blood biomarkers across independent cohorts, longitudinal assessments of these potential biomarkers have not previously been carried out. Therefore, candidate genes from three cross-sectional studies were evaluated in our longitudinal analysis cohort to provide further evaluation of their suitability as Huntington's disease biomarkers (Supplementary Table 10). It would be expected that if a gene is differentially expressed between controls and Huntington's disease in these studies, it would be correlated with disease progression in the same direction.

Of the same 70 transcripts described above, the expression of only four changed at a rate that could be statistically significantly associated with disease progression over the 2-year interval ($P < 0.05$ without FDR correction). These are plotted in Fig. 3, and Table 3 summarizes their functions and correlation statistics.

ROCK1, which encodes a protein serine/threonine kinase (Borovecki et al., 2005), showed the strongest correlation of any of the previously identified candidate biomarkers in our longitudinal analysis. To the contrary, however, it was not detected as differentially expressed in our cross-sectional differential gene expression analyses (nominal $P > 0.05$), where it showed a trend in the opposite direction.

SAP30 also showed an upregulation over the 2-year interval that was significantly associated with disease progression. This corroborates the findings of (Zadel et al., 2018) and was also detected in the cross-sectional analysis our Year 1 samples.

Likewise, *LTBR* showed significant positive correlation with TMS progression, consistent with a previous study of NF κ B pathway disturbances in Huntington's disease (Träger et al., 2014). *LTBR* also showed upregulation (nominal $P < 0.05$) in our Year 3 differential expression analysis.

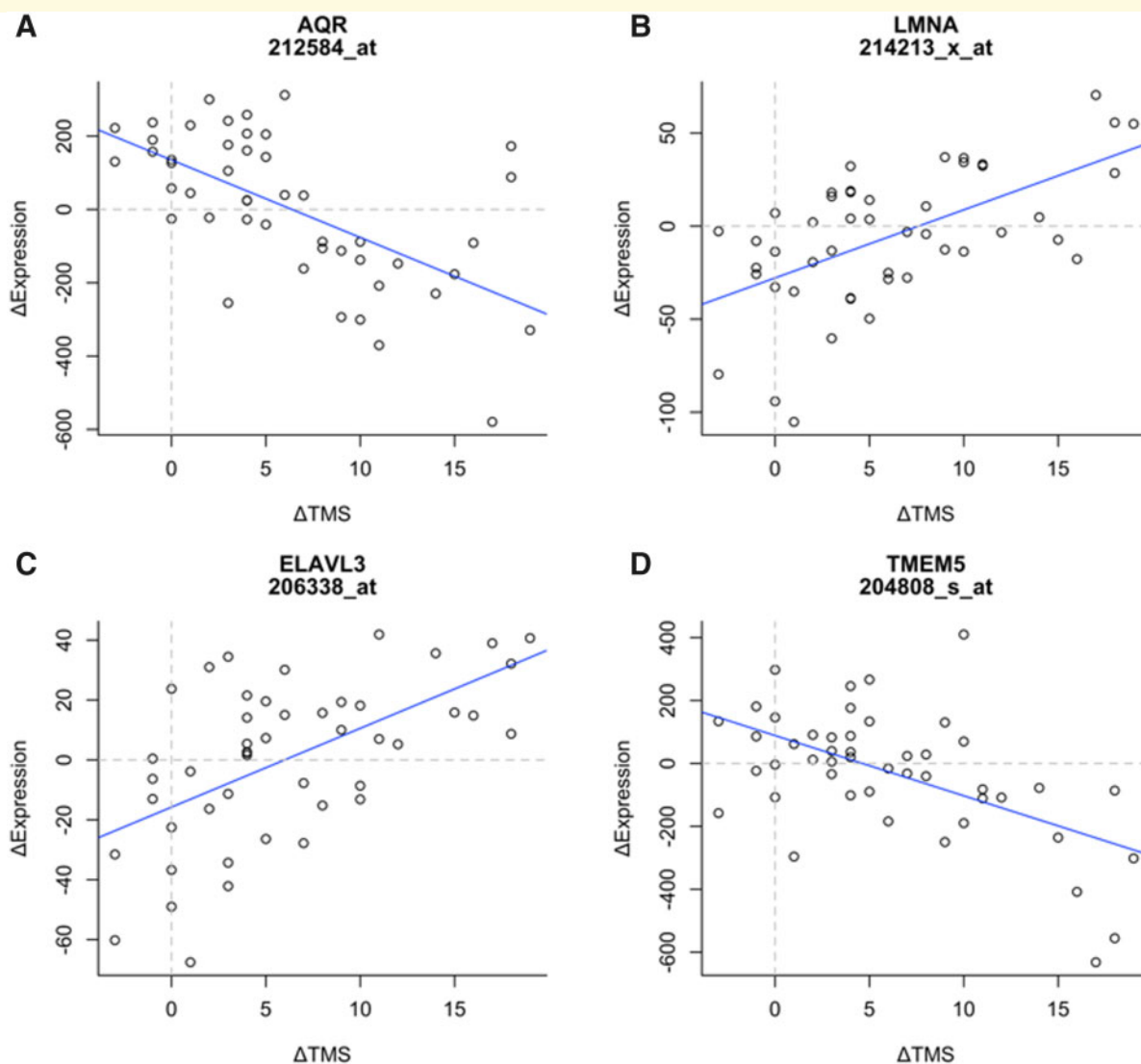


Figure 2 The four probesets with the strongest correlation between disease progression and differential expression over 2 years. These analyses are based on data from preHD B ($n = 15$; mean age at Year 3 \pm SD: 39.9 ± 8.9 years; mean TMS change \pm SD: 4.67 ± 4.47) and diagnosed Huntington's disease subjects zHD stage 1 ($n = 16$; 50.0 ± 9.3 years; 6.56 ± 6.74) and zHD stage 2 ($n = 16$; 54.5 ± 6.6 ; 7.13 ± 6.16). Correlation calculated using Pearson's correlation coefficient (r). When the significance of correlation was calculated and multiple testing correction performed, adjusted $P > 0.05$ in all probesets. **(A)** AQR: $r = -0.61$; nominal $P = 4.3 \times 10^{-6}$. **(B)** LMNA: $r = +0.58$; nominal $P = 1.6 \times 10^{-5}$. **(C)** ELAVL3: $r = +0.58$; nominal $P = 2.3 \times 10^{-5}$. **(D)** TMEM5: $r = -0.56$; nominal $P = 4.9 \times 10^{-5}$.

The other gene showing significant correlation with disease progression was *IL23A*, which encodes an inflammatory cytokine. However, *IL23A* was reported to be upregulated in the original paper (Miller *et al.*, 2016), but showed negative association with disease progression in our analysis.

Functional pathway analyses of a putative Huntington's disease signature

Several studies have interrogated specific functional pathways represented in gene expression signatures from

Huntington's disease blood using GSEA. In their analysis of Huntington's disease monocytes, Miller *et al.* (2016) demonstrated significant enrichment of upregulated genes relating to inflammation, immunity and intracellular signalling, and downregulated genes relating to vacuolar and catabolic functions.

Hensman Moss *et al.* (2017) conducted a GSEA of differentially expressed genes from a cohort of 186 Huntington's disease and preHD subjects and 49 control blood samples, a subset of which were also analysed in this study. They reported significant associations of RNA measures with TMS that represented pathways similar to those reported by Miller *et al.* (2016). However, as in

Table 2 Top 20 probesets with strongest correlation longitudinal analysis of gene expression and TMS progression

| Gene symbol | Probeset | Protein function | Correlation | P-value |
|-------------|--------------|--|-------------|---------|
| AQR | 212584_at | Intron-binding spliceosomal factor | -0.61 | 4.3E-06 |
| LMNA | 214213_x_at | Component of nuclear lamina | 0.58 | 1.6E-05 |
| ELAVL3 | 206338_at | Cell differentiation | 0.58 | 2.2E-05 |
| TMEM5 | 204808_s_at | Transmembrane protein | -0.56 | 5.0E-05 |
| HSPA9 | 200690_at | Stress response, mitochondrial maintenance | -0.55 | 5.4E-05 |
| (EST) | 242526_at | (Unknown) | -0.55 | 6.6E-05 |
| NACCI | 235047_x_at | Apoptosis, transcriptional regulation | 0.55 | 7.2E-05 |
| PRX | 220024_s_at | Axon ensheathment | 0.54 | 8.2E-05 |
| LINC00691 | 1561059_a_at | (Non-protein coding) | 0.54 | 1.1E-04 |
| IGFBP1 | 237989_at | IGF-binding protein | 0.53 | 1.1E-04 |
| (EST) | 239312_at | (Unknown) | 0.53 | 1.2E-04 |
| (EST) | 220129_at | (Unknown) | 0.53 | 1.4E-04 |
| MGC16025 | 1553747_at | (Non-protein coding) | 0.53 | 1.5E-04 |
| LYPD6 | 227763_at | Interacts with nAChR subunits | 0.52 | 1.6E-04 |
| MAPRE3 | 203841_x_at | Regulation of microtubule cytoskeleton | 0.52 | 1.8E-04 |
| TSGA10 | 223838_at | (Unknown) | -0.52 | 1.9E-04 |
| (EST) | 237213_at | (Unknown) | -0.52 | 2.0E-04 |
| CCDC150 | 1553459_at | (Unknown) | 0.52 | 2.1E-04 |
| (EST) | 1557505_a_at | (Unknown) | 0.51 | 2.2E-04 |
| CNTNI | 1554784_at | Neuronal cell adhesion | 0.51 | 2.5E-04 |

These analyses are based on data from 15 preHD B and 32 diagnosed Huntington's disease subjects. Correlations were calculated using Pearson's correlation coefficient. *P*-values have not been adjusted. EST and signifies microarray tags for which the gene they represent is currently unknown. Protein function from GeneCards Human Gene Database.

our study, they did not observe statistically significant differential expression in individual genes between Huntington's disease subjects and controls.

We first conducted GSEAs of differentially expressed genes across our entire Year 1 cohort. Of the 5519 gene sets tested, none were found to be statistically significantly overrepresented (*q*-value cutoff of 0.05) (Supplementary Tables 11 and 12). We then repeated the analysis with the exclusion of samples in the presymptomatic groups (as we did not observe statistically significant difference in mean TMSs in this group compared to controls). Again, we did not observe any significant enrichment of pathways (Supplementary Tables 13 and 14). We discuss possible biological and technical explanations for these discrepancies below.

Discussion

In this study, we aimed to identify a transcriptional signature in whole blood that could be used as a Huntington's disease state biomarker, and to evaluate candidate RNA biomarkers identified in previous studies. These aims were pursued through differential expression analyses of blood samples from manifest Huntington's disease subjects, presymptomatic subjects carrying the Huntington's disease mutation and control subjects. Both cross-sectional and longitudinal analyses were applied and correlated with UHDRS measures. To our knowledge, this is the first time a longitudinal analysis in a single Huntington's disease cohort has been used to try to identify novel transcriptional Huntington's disease biomarkers and to test previously reported candidates.

The high level of variability in blood RNA expression may be a substantial barrier to the clinical application of gene expression changes as clinical biomarkers. Here, differential expression in Huntington's disease subjects versus controls was investigated using multiple linear regression modelling, to attempt to assign potential confounding effects of collection site, gender and age *a priori*. The *P*-values discussed here are not adjusted for multiple testing, because adjustments using the standard FDR method resulted in *P*-values greater than 0.05 and therefore would not have aided us in discriminating top candidates. This likely reflects the disease effect sizes being very small, particularly considering the available sample numbers and other variables (Ideker *et al.*, 2011). Quality control measures of the microarray data showed low technical variability, which was thus ruled out as a confounding factor.

Furthermore, our pathway analyses did not show convincing evidence of dysregulation of any particular functional cellular or molecular pathways. This does not exclude the possibility of functional pathway dysregulation (Hensman Moss *et al.*, 2017), but differences from previous approaches and their caveats need to be considered. Here, we randomized the disease status assignments of the samples to evaluate the background signal in our analyses. These maintain the data structure of the defined background, thereby circumventing confounding effects due to tissue-specific gene expression. For example, in blood, signatures are dominated by immune cell and reticulocyte RNAs, which would typically represent specific biological pathways compared to randomly sampled gene sets. In such a case, false-positive assignments of disease-

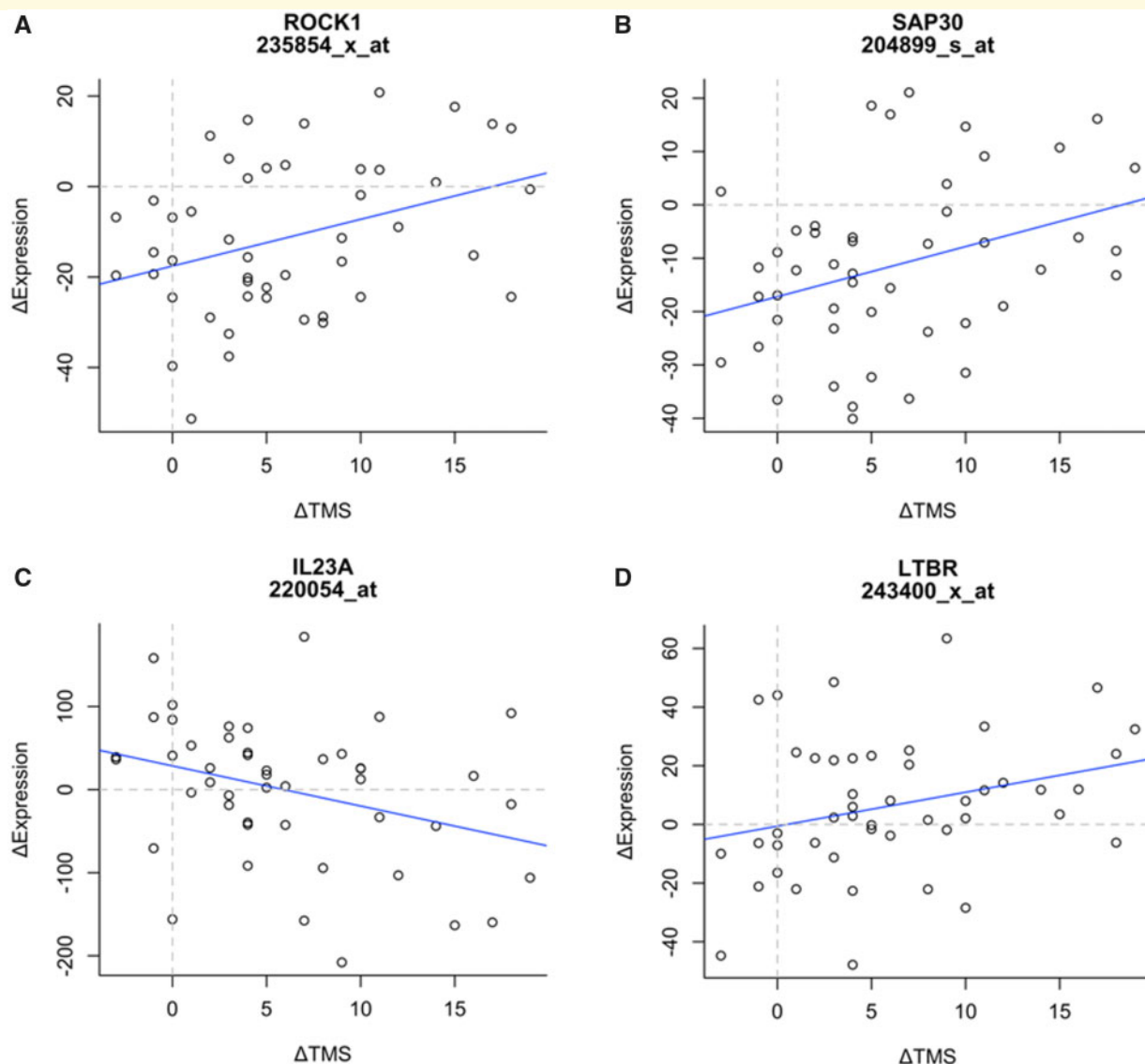


Figure 3 Probesets representing genes found to be dysregulated in previously transcriptomic Huntington's disease blood studies that showed significant association between disease progression and differential expression in our longitudinal analysis.

These analyses are based on data from preHD B ($n = 15$; mean age at Year 3 \pm SD: 39.9 ± 8.9 years; mean TMS change \pm SD: 4.67 ± 4.47) and diagnosed Huntington's disease subjects zHD stage 1 ($n = 16$; 50.0 ± 9.3 years; 6.56 ± 6.74) and zHD stage 2 ($n = 16$; 54.5 ± 6.6 ; 7.13 ± 6.16). Correlation calculated using Pearson's correlation coefficient (r). When the significance of correlation was calculated and multiple testing correction performed, adjusted $P > 0.05$ in all probesets. **(A)** ROCK1: $r = +0.36$; nominal $P = 0.013$. **(B)** SAP30: $r = +0.35$; nominal $P = 0.017$. **(C)** IL23A: $r = -0.33$; nominal $P = 0.022$. **(D)** LTBR: $r = +0.29$; nominal $P = 0.049$.

related differential gene expression to pathways of immune cell signalling and inflammation become more likely.

We also evaluated the relationship between differential expression attributed to Huntington's disease and continuous quantitative measures of disease status (UHDRS TMS) using linear correlation. The slopes of the resultant lines were also low, further supporting a small Huntington's disease effect size. The small effect size could however reflect the proportion of blood cell

subtypes susceptible to Huntington's disease-related changes (Björkqvist *et al.*, 2008; Wild *et al.*, 2011).

We nonetheless entertained the possibility that small transcriptional changes in peripheral blood cells might be informative as state biomarkers in individual Huntington's disease patients. However, longitudinal analyses of the 70 genes identified by previous cross-sectional studies [not including those from Hensman Moss *et al.* (2017), whose cohort overlapped with our own] detected only three that showed significant association

Table 3 Functions of four probesets representing genes identified in previous studies as dysregulated in Huntington's disease blood and their association with disease progression calculated in our longitudinal analysis of gene expression and TMS

| Gene symbol | Probeset | Protein function | Correlation | P-value |
|--------------|-------------|---|-------------|---------|
| <i>ROCK1</i> | 235854_x_at | Regulation of actomyosin cytoskeleton | 0.36 | 0.013 |
| <i>SAP30</i> | 204899_s_at | Histone deacetylase complex subunit | 0.35 | 0.017 |
| <i>IL23A</i> | 220054_at | Innate and adaptive immunity | -0.33 | 0.022 |
| <i>LTBR</i> | 243400_x_at | Lymphoid development, lipid metabolism, apoptosis | 0.29 | 0.049 |

Samples included in the analysis were those of 15 preHD B individuals and 32 diagnosed Huntington's disease subjects. Correlation calculated using Pearson's correlation coefficient. P-values have not been adjusted. Protein function from GeneCards Human Gene Database.

with disease state in the same direction (*ROCK1*, *SAP30*, *LTBR*). The very minimal overlap of Huntington's disease-associated gene expression changes from previous studies in our 2-year longitudinal analyses in does not bode well for their suitability as biomarkers. Again, the most likely explanation is that the small Huntington's disease effect size is insufficient compared to the inherent biological and technical variability of blood RNA differential expression measures.

The functions of the proteins encoded by genes with the most reproducible differential expression in Huntington's disease samples are nonetheless consistent with idea that mHTT has pathological effects in blood. For example, *ROCK1* is a member of the Rho kinase pathway and is involved in cell motility and cytoskeleton organization. This pathway has previously been documented to be dysregulated in Huntington's disease blood, post-mortem brain samples and mouse models (Narayanan *et al.*, 2016).

SAP30 is a component of the histone deacetylase complex, and its dysregulation may relate to changes in the post-translational modification of histone proteins, which are known to occur in Huntington's disease and Huntington's disease model systems (Glajch and Sadri-Vakili, 2015; Naia *et al.*, 2017). Likewise, dysregulation of *LTBR*, a receptor involved in cytokine release, contributes to the wider evidence of dysregulated inflammation in Huntington's disease (Miller *et al.*, 2016; Hensman Moss *et al.*, 2017). Clearly, these changes and their functional consequences could help understand Huntington's disease pathology in peripheral tissues, and how this differs from, or is similar to, mHTT's effects in the brain (Weir *et al.*, 2011).

Intra- and inter-individual variation and the potential for uncorrelated peripheral and central pathologies seem to be at the root of difficulties discerning a temporal association between blood gene expression and the neurological symptoms of Huntington's disease. In contrast, other biomarker approaches may prove more useful. These include neuroimaging (Rees *et al.*, 2013), alternative measures of motor function (e.g. speeded tapping, tongue force and eye movements) (Tabrizi *et al.*, 2009) and other molecular analyses, including measurements of neurofilament proteins (Byrne *et al.*, 2018; Johnson *et al.*, 2018), HTT protein (Wild *et al.*, 2015; Byrne *et al.*,

2018), oxidative stress markers, inflammatory proteins and endocrine markers (Björkqvist *et al.*, 2008; Wild *et al.*, 2011; Scahill *et al.*, 2012).

In conclusion, changes in mRNA expression in whole blood showed limited association with Huntington's disease pathology and progression. These changes may reflect interesting pathological or compensatory roles within blood cells, but their effect sizes in whole blood are very small, thereby constraining their deployment as state biomarkers for Huntington's disease.

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Supplementary material

Supplementary material is available at *Brain Communications* online.

Competing interests

The authors report no competing interests.

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