BRAIN COMMUNICATIONS

Gut dysbiosis in Huntington's disease: associations among gut microbiota, cognitive performance and clinical outcomes

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Huntington's disease is characterized by a triad of motor, cognitive and psychiatric impairments, as well as unintended weight loss. Although much of the research has focused on cognitive, motor and psychiatric symptoms, the extent of peripheral pathology and the relationship between these factors, and the core symptoms of Huntington's disease, are relatively unknown. Gut microbiota are key modulators of communication between the brain and gut, and alterations in microbiota composition (dysbiosis) can negatively affect cognition, behaviour and affective function, and may be implicated in disease progression. Furthermore, gut dysbiosis was recently reported in Huntington's disease transgenic mice. Our main objective was to characterize the gut microbiome in people with Huntington's disease and determine whether the composition of gut microbiota are significantly related to clinical indicators of disease progression. We compared 42 Huntington's disease gene expansion carriers, including 19 people who were diagnosed with Huntington's disease (Total Functional Capacity > 6) and 23 in the premanifest stage, with 36 age- and gender-matched healthy controls. Participants were characterized clinically using a battery of cognitive tests and using results from 16S V3 to V4 rRNA sequencing of faecal samples to characterize the gut microbiome. For gut microbiome measures, we found significant differences in the microbial communities (beta diversity) based on unweighted UniFrac distance ($P = 0.001$), as well as significantly lower alpha diversity (species richness and evenness) between our combined Huntington's disease gene expansion carrier group and healthy controls ($P = 0.001$). We also found major shifts in the microbial community structure at Phylum and Family levels, and identified functional pathways and enzymes affected in our Huntington's disease gene expansion carrier group. Within the Huntington's disease gene expansion carrier group, we also discovered associations among gut bacteria, cognitive performance and clinical outcomes. Overall, our findings suggest an altered gut microbiome in Huntington's disease gene expansion carriers. These results highlight the importance of gut biomarkers and raise interesting questions regarding the role of the gut in Huntington's disease, and whether it may be a potential target for future therapeutic intervention.

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Graphical Abstract

Introduction

Huntington's disease is an inherited neurodegenerative disease that causes progressive motor decline, cognitive dysfunction and neuropsychiatric symptoms (Walker, 2007). Huntington's disease is caused by age-dependent penetrance of an expanded sequence of cytosine–adenine–guanine (CAG) repeats in the *huntingtin* gene (Tabrizi *et al.*, 2011). Larger expansions of CAG repeats are associated with earlier disease onset and more rapid disease progression (Andrew *et al.*, 1993). Across the disease course, Huntington's disease affects a wide range of functions, including communication, awareness, executive functioning, organization, memory and visuospatial abilities, all of which decline over time (Craufurd *et al.*, 2001; Berrios *et al.*, 2002; Paulsen *et al.*, 2008; Ross and Tabrizi, 2011). Cognitive decline eventually progresses to dementia, and furthermore, depression and suicide are estimated to be 5–10 times more common in Huntington's disease than in the general population (Berrios *et al.*, 2002; Walker, 2007). In the brain, Huntington's disease affects the basal ganglia early and severely, but the brain effects of Huntington's disease progress and generalize to a range of cortical and subcortical structures, affecting both grey and white matter (Tabrizi *et al.*, 2009, 2011; Domínguez *et al.*, 2016).

These brain changes have been assumed to account for the clinical manifestations seen in Huntington's disease (Ross and Tabrizi, 2011), however, peripheral pathology is an underexplored area of investigation.

In addition to the cognitive, motor and neuropsychiatric symptoms, which likely relate to brain changes, people with Huntington's disease also experience a range of gastrointestinal disturbances, including diarrhoea, nutrient deficiencies, gastritis and unintended weight-loss, which are considered clinical features of Huntington's disease (Djousse *et al.*, 2002; Robbins *et al.*, 2006; van der Burg *et al.*, 2017). Some evidence suggests that these disturbances are a manifestation of dysfunction in the gastrointestinal tract (van der Burg *et al.*, 2011). In addition, gastrointestinal disturbances may be indirect effects of other progressive disease features, such as cognitive decline and decreased mobility. Whilst the majority of research investigating the pathogenesis of Huntington's disease has focused on brain atrophy and the accompanying cognitive, behavioural and psychiatric symptoms, the extent to which peripheral pathology, and in particular the gut, is related to core symptoms of Huntington's disease remains relatively unknown (Kong *et al.*, 2018).

Adding to this picture, extensive evidence highlights the relationship among the enteric nervous system, the gut microbiome and the brain, which are collectively referred

to as the gut–brain axis (Carabotti *et al.*, 2015). The gut–brain axis is a bidirectional chemical signalling system between the gastrointestinal system and the central nervous system (Burokas *et al.*, 2015). Furthermore, alterations of the gut microbiome influence mood regulation, cognition and sleep (Cryan and Dinan, 2012), and increasing evidence suggests that diversity in the gut microbiome is essential for brain function (Dinan and Cryan, 2017). The recognition of the functional significance of the gut–brain axis is increasingly influential, and has even been argued to be a paradigm shift in neuroscience (Mayer *et al.*, 2014). With the rise in research activity on this topic, a substantial, growing body of evidence now links the gut–brain axis to neurodegenerative, neurodevelopmental and neuropsychiatric diseases (Marques *et al.*, 2016; Sarkar and Banerjee, 2019).

Altered gut microbiome profiles have been documented in several neurodegenerative diseases, such as multiple sclerosis (Newland *et al.*, 2016), Parkinson's disease (Scheperjans *et al.*, 2015), amyotrophic lateral sclerosis (Fang *et al.*, 2016) and Alzheimer's disease (Vogt *et al.*, 2017; for a full review refer to Sarkar and Banerjee, 2019). The most abundant evidence of gut dysbiosis is in Parkinson's disease (Keshavarzian *et al.*, 2015; Scheperjans *et al.*, 2015; Fitzgerald *et al.*, 2019). In Parkinson's disease, alterations in gut microbiota are thought to promote α-synuclein-mediated motor deficits and neuroinflammation, which specifically connects the gut–brain axis to disease pathogenesis (Sampson *et al.*, 2016; Fitzgerald *et al.*, 2019). Furthermore, transplants of gut microbiota from humans with Parkinson's disease into mice, exacerbate motor deficits, underlining the interplay between gut microbiota and the progression of disease (Sampson *et al.*, 2016). People with Parkinson's disease also exhibit dysbiosis, and a greater abundance of *Enterobacteriaceae* correlates with severity of motor signs, including postural instability and gait abnormalities (Scheperjans *et al.*, 2015). Parkinson's disease, as well as other neurodegenerative diseases, is linked to lower than normal levels of *Eubacterium hallii* and *Prevotella* (Louis *et al.*, 2010; Engels *et al.*, 2016; Gerhardt and Mohajeri, 2018). These bacteria are involved in the production of short-chain fatty acids, which play a major role in intestinal homeostasis, gut and colon function (Bourassa *et al.*, 2016), and may be a mechanism underlying some of the gut signs and symptoms observed in Parkinson's disease.

Compared to the extensive evidence emerging in Parkinson's disease, no gut microbiome studies have been reported in people with Huntington's disease, despite intriguing findings from Huntington's disease animal models (Kong *et al.*, 2018; Radulescu *et al.*, 2019). For example, studies in Huntington's disease mouse models show gut dysfunction, along with evidence of endocrine dysfunction, including hormonal abnormalities, decreased enteric neuropeptides, decreased mucosal thickness and villus length (Ferrante *et al.*, 2000; van der Burg *et al.*,

2011; McCourt *et al.*, 2015). Furthermore, deficient energy metabolism, as well as serum and cerebrospinal fluid metabolic differences, has been observed in presymptomatic transgenic Huntington's disease rats (Verwaest *et al.*, 2011). More recently, in R6/1 transgenic mice, Kong *et al.* (2018) observed the first specific evidence of gut dysbiosis in a Huntington's disease model, finding increases in Bacteroidetes and a proportional decrease in Firmicutes. In this study, gut dysbiosis was also associated with body weight impairment and motor deficits.

Given the preclinical evidence suggesting gut dysbiosis in Huntington's disease models and clinical evidence of gut symptoms in Huntington's disease, together with the emerging picture of gut dysbiosis in neurodegenerative conditions, in this study we used faecal samples to investigate whether the gut microbiome in Huntington's disease gene expansion carriers (HDGECs) differs from age- and gender-matched healthy controls (HCs). We sought to investigate differences in terms of the *richness and evenness* of diversity of the gut, which confers resilience of gut function, the *composition of microbial communities*, as indicated by taxonomic analysis and the various Phyla and Families of bacteria constituting compositional differences, and *the functional pathways and enzymes* associated with these gut profiles. We also examined the relationship among Phyla, Family and certain species of bacteria, with clinical measures of motor and cognitive function.

Materials and methods

Participants

We studied 42 HDGECs, including 19 people who were diagnosed with Huntington's disease, 23 people in the premanifest stage and 36 HCs. All premanifest HDGECs had been genetically confirmed to have the Huntington's disease CAG expansion (i.e. CAG $\geq$ 39; except for one premanifest HDGEC close to manifest diagnosis). For the Huntington's disease sample, diagnosis was made by a neurological examination and the neurologist's classification of the participant as clinically symptomatic, based on unequivocal motor signs of Huntington's disease using the Unified Huntington's Disease Rating Scale (UHDRS; Kieburtz *et al.*, 2001). We used scaled CAG-age product (CAP$_S$) scores to classify our premanifest HDGEC participants based on a 5-year probability of symptomatic diagnosis into cut-offs of low probability, medium probability and high probability, based on the optimization algorithm from the PREDICT-HD study (Epping *et al.*, 2013). CAP$_S$ is calculated by multiplying the age at time of testing (Age$_0$) by scaling the CAG repeat length, where CAP$_S$ = Age$_0$ × (CAG - 33.66)/432.3326 (Zhang *et al.*, 2011). We had seven premanifest participants in the low cut-off (CAP$_S$ < 0.67), seven participants in the medium cut-off (CAP$_S$ between 0.67 and 0.85), and seven

participants in the high cut-off ($CAP_S > 0.85$). We could not calculate CAP_S for two participants because of missing genetic data.

We recruited participants from the Statewide Progressive Neurological Disease Service at Calvary Health Care Bethlehem Hospital and the Experimental Neuropsychology Research Unit and Clinical Cognitive Neuroscience Huntington's disease research participant database at Monash University, Melbourne, Australia. Exclusion criteria for all participants included diagnosis by a general practitioner or medical professional of irritable bowel syndrome, coeliac disease, diabetes or history of any diagnosed condition that may likely influence general gut health (e.g. Crohn's disease and ulcerative colitis). Furthermore, participants were excluded if they had psychiatric illness (except for psychiatric symptoms attributable to Huntington's disease), neurological illness (except for Huntington's disease), a history of traumatic brain injury, recent antibiotic or anti-inflammatory medication use (past 2 months) or current recreational substance use.

Sample characterization measures

Depression is prevalent in Huntington's disease (Walker, 2007), and can negatively affect cognition and the gut microbiome. We therefore screened participants using the Center for Epidemiologic Studies Depression Scale Revised (Eaton *et al.*, 2004). Center for Epidemiologic Studies Depression Scale Revised scores range from 0 to 80, with higher scores indicative of greater depressive symptoms. As the focus of this study was on the gut microbiome, we wanted to consider gastrointestinal symptoms. Therefore, we included a clinically used measure of gut health, the self-report Gastrointestinal Health Appraisal Questionnaire (Metagenics®, 1992). The Gastrointestinal Health Appraisal Questionnaire includes 35 questions relating to gastric functioning, gastrointestinal inflammation, functioning of the small intestine and pancreas and colon function. Gastrointestinal Health Appraisal Questionnaire scores range from 0 to 280, with higher scores indicative of higher gastrointestinal symptoms. We administered the Wechsler Test of Adult Reading (Holdnack, 2001) to estimate participants' premorbid intelligence. The Wechsler Test of Adult Reading comprises a list of 50 words that do not follow typical pronunciation rules, and participants are required to read the words aloud. A higher score (i.e. larger number of correctly pronounced items) reflects a higher estimated premorbid intelligence quotient score. On these measures, groups did not statistically differ on depressive symptoms, self-reported gastrointestinal symptoms or estimated premorbid intelligence.

For the HDGECs, the UHDRS (Kieburtz *et al.*, 2001) Total Motor Score (TMS) and Total Functional Capacity (TFC) components were used to characterize severity of motor signs and the stage of disease. Higher TMS indicates more severe motor signs. The TFC is a clinician

Table 1 Demographic and clinical profile of HDGEC and HC participants

		HDGEC = 42	Control = 36	P/χ^2 value
Gender	M:F	22:20	15:21	0.345
Age (years)	M (SD)	50.16 (12.14)	50.55 (13.90)	0.948
	Range	26–75	24–69	
UHDRS–TMS	M (SD)	9.21 (12.71)		
	Range	0–41		
UHDRS–TFC	M (SD)	10.90 (2.64)		
	Range	6–13		
Disease duration (years after diagnosis)	M (SD)	5.06 (3.23)		
	Range	1–13		
CAG length	M (SD)	41.26 (1.80)		
	Range	38–46		
CAP_S	M (SD)	0.88 (0.25)		
	Range	0.32–1.40		
Years of education	M (SD)	14.61 (2.94)	14.83 (3.20)	0.461
	Range	8–21	9–23	
Estimated IQ	M (SD)	104.04 (6.99)	106.14 (6.43)	0.171
	Range	87–115	89–117	
GIHAQ	M (SD)	21.47 (25.44)	15.81 (21.33)	0.294
	Range	0–119	0–116	
CESD-R	M (SD)	10.92 (10.45)	7.06 (7.75)	0.071
	Range	0–43	0–31	

CAP_S: scaled CAG-age product score (for premanifest HDGECs only); estimated intelligence quotient (IQ) calculated by Wechsler Test of Adult Reading; CESD-R: Center for Epidemiologic Studies Depression Scale-Revised (range: 0–80); F: female; GIHAQ: Gastrointestinal Health Appraisal Questionnaire (range: 0–280); M: male; TFC: Total Functional Capacity (range: 0–13); disease duration is for manifest HDGECs only; TMS: Total Motor Score (range: 0–124). P/χ^2 value signifies probability value between groups, using independent samples *t*-tests or Chi-squared test (χ^2 for gender).

rating scale including items related to capacity to work, handle finances, perform domestic chores, self-care and ability to live independently (Shoulson and Fahn, 1979). These ratings yield a TFC score, with higher scores indicating better functioning and greater independence. The TFC and TMSs were obtained from each HDGEC participant's treating neurologist/clinician. A TMS for three participants was unavailable. Demographic and clinical features of the participant groups are presented in Table 1. Supplementary Table 1 contains demographic and clinical information of HDGEC participants separated by disease status (i.e. premanifest or manifest).

Ethical approval

Monash University Human Research Ethics Committee approved this study (MUHREC: 8031). All participants provided written informed consent in accordance with the Declaration of Helsinki (World Medical Association, 2013).

Procedure

The data reported in this study were collected as baseline data for a probiotic clinical trial, which was ongoing

when this manuscript was prepared. Participants were screened for eligibility by phone, and then scheduled for a 90-min assessment session in the lab. To enable the collection of a faecal sample, participants were mailed a stool collection kit in advance. The kit included instructions on collecting and storing the stool sample, as well as collection tubes, latex gloves, plastic containers and sealable plastic bags. To enable the assessment of a panel of gut bacteria, participants provided a faecal sample within 24 h of their in-lab testing session, which they stored on ice until attending the testing session. Once participants arrived at the testing session, we stored their stool sample at −80°C for later processing. We then administered a battery of neuropsychological tasks and a measure used to estimate premorbid intelligence. Participants also filled out self-report measures of depression and gastrointestinal function. We reimbursed participants for their time at the end of the testing session.

Measures

Cognitive measures

We used cognitive measures from the Huntington's Disease Cognitive Assessment Battery (HD-CAB), which is a battery specifically designed for cognitive assessment in Huntington's disease clinical trials (Stout et al., 2014). HD-CAB includes six cognitive tests. We describe each task below; for more details refer to Stout et al. (2014).

The Hopkins Verbal Learning Test-Revised (Benedict et al., 1998) is a paper and pencil task that assesses verbal learning and memory (immediate and delayed recall). On this task, participants must recall as many items as they can from a word list, during three learning trials and the delayed recall trial. The main outcome measure is total number of words recalled across the three trials summed with the number of words recalled on the delayed trial.

The Symbol Digit Modalities Test measures processing speed and visual attention (Smith, 1982). Participants must refer to a key and fill in each empty box with a number that correctly corresponds to each symbol, as quickly as they are able to within 90 s. The outcome measure is the total numbers of boxes correctly completed.

The Trail Making Test (Reitan, 1958) parts A and B measures processing and manual movement speed, visuo-spatial attention and executive functioning. The outcome measure we used for this task is the time taken (in seconds) to complete Trail Making Test part B, where participants are required to draw a continuous line connecting numbers and letters in alteration (1 to A, A to 2, 2 to B, etc.). Longer times are indicative of poorer performance.

Paced tapping (Rowe et al., 2010; Stout et al., 2014) is a computerized task used to measure psychomotor coordination and timing. Participants are instructed to tap a mouse using alternating thumbs at a consistent rhythm.

They complete four identical trials. The outcome measure for this task is the reciprocal of the standard deviation of the inter-tap interval, which measures the overall consistency of the tapping rate. Higher values indicate better performance.

The Emotion Recognition Task (Johnson et al., 2007) is a computerized measure of participants' ability to recognize emotions in facial expressions. Participants are presented with black and white photographed faces that depict seven emotional expressions: anger, disgust, fear, happiness, sadness, surprise or neutral. The outcome measure is the correct number of negative emotions identified (anger, disgust, fear and sadness). Higher scores are indicative of greater accuracy.

The One Touch Stockings of Cambridge (OTS) is a modified, shortened version (6Touch10) of the CANTAB® OTS task. OTS is a computerized task that measures working memory and spatial planning (Robbins et al., 1994; Watkins et al., 2000). Participants have to imagine moving balls (one at a time), to make the bottom arrangement of balls match the top, in as few moves as possible. During each trial, participants are instructed to select the minimum numbers of moves (from one to six) required to make the bottom set match the top set. The outcome measure is the mean time to reach a correct response (seconds), averaged across all trials. Higher values are indicative of poorer performance.

Using the six tests included in the HD-CAB, we computed a composite score for a cognitive outcome measure. We first rescaled Trail Making Test part B and OTS so that higher scores represented better performance. For these variables, all values were multiplied by −1 and then a constant value (highest value for each variable) was added to all values. We then computed an equal-weighting composite score by adding the average standardized Z-score for each individual test (Hopkins Verbal Learning Test-Revised, Symbol Digit Modalities Test, Trail Making Test part B, Paced tapping, Emotion Recognition and OTS). We also computed separate equal-weighting cognitive composite scores, standardized from only our HDGEC group, for certain analyses restricted to the HDGEC group.

Faecal DNA extraction, sequencing and pre-processing. We extracted a 200–250 mg aliquot from each frozen faecal sample and placed them in 2 ml bead extraction tubes. The bead extraction tubes were stored on dry ice and couriered to the Australian Genomic Research Facility in Adelaide, South Australia, for bacterial genomic DNA extraction. All samples included in this report, were extracted at a single time-point to eliminate variation in results because of possible batch effects. The 16S V3–V4 rRNA gene (341F–806 R), which is a hypervariable region of bacterial rRNA for tracing phylogeny, was amplified and the final concentrations were determined by fluorometry. All aliquots included had > 0.50 ng/µl of

usable amplicon, and were deemed sufficient for the sequencing stage. Amplicon was pooled and sequenced using an Illumina MiSeq next-generation sequencer. The reads were then demultiplexed and quality trimmed before denoizing using default parameters on DADA2 v1.12 in R (Callahan *et al.*, 2016) to obtain the amplicon sequence variants (ASVs) table. Samples that did not have at least 1000 reads were not included in our analyses. The ASVs were mapped against SILVA 132_99 database (Quast *et al.*, 2012) to obtain taxonomic identity of the reads. Results from this process were provided as a tabulation of the relative abundance of bacteria at the Phylum and Family levels. The full dataset of results received from the analyses included bacteria from seven Phyla, 32 Families and 79 Genera. The majority of the bacterial Phyla across the samples were Firmicutes (83%), Actinobacteria (9%), Bacteroidetes (4%) and Verrucomicrobia (1.1%).

Determination of key outcome measures from faecal samples. To characterize *richness*, *evenness* and *composition of the microbiome environment*, we assessed *alpha and beta diversity*. We used the number of ASVs observed, as well as the Fisher index, to determine richness and evenness, i.e. alpha diversity of each participant's microbial community, and unweighted UniFrac distances for group environment composition, i.e. beta diversity. For alpha diversity measures, the reads were rarefied to 1984 reads, which is the global minimum number of reads in the cohort. For beta diversity, unweighted UniFrac distances were calculated and ordinated in principal coordinated analysis to visualize the variation in the data. The unweighted UniFrac distance accounts for phylogenetic relationship between ASVs. Adonis (Permutation multivariate ANOVA) from the 'vegan' v2.5.6 R package was performed with 999 permutations (Dixon, 2003). Adonis provides an R^2 value on a scale of 0 to 1, indicating the amount of variance explained by the factors tested. Both alpha and beta diversity were analysed using the 'Phyloseq' v1.28 R package (McMurdie and Holmes, 2013). For examining *differences which describe observed differences in the composition* of the microbial environment, we used taxonomic analysis at the Phylum and Family level. Rare bacterial families were filtered using the cut-off of minimum 10 reads per sample, resulting in 29 bacterial Families altogether and 7 Phyla.

Gut function analysis using PICRUSt2 to identify functional pathways and enzymes. Raw sequences were passed through PICRUSt2 workflow to obtain enzyme commission numbers and Kyoto Encyclopedia of Genes and Genomes Ortholog abundances, which describe potential disturbance in gut microbiome enzymes and functional pathways, respectively (Douglas *et al.*, 2020). As with the microbiome composition analysis, alpha and beta diversity of Kyoto Encyclopedia of Genes and Genomes Ortholog abundances and enzyme commission numbers were analysed using Phyloseq in R.

Statistical analysis

We used the Kruskal–Wallis test to compare species richness and alpha-diversity measurements between groups, and Adonis Permutation multivariate ANOVA to compare microbial composition (beta diversity indices) between groups. For examining taxonomies, we used analysis of composition of microbiomes, implemented in QIIME2 v2019.7 (Caporaso *et al.*, 2010) to test for differential abundances of bacteria between the HDGEC and HC groups in the Phylum and Family levels (Mandal *et al.*, 2015). Analysis of composition of microbiomes is shown to control for false-discovery rates while maintaining high power and is much better suited for analysing microbiome relative abundance data, compared to other standard statistical tests such as *t*-test and ANOVA (Mandal *et al.*, 2015). The W-statistic value outputted by analysis of composition of microbiomes indicates the number of times the null hypothesis has been rejected for each ASV, with a false-discovery rate of < 0.05. For all analyses of group differences (HDGECs versus HC) we controlled for gender effects. To compare functional pathways and enzymes between groups, we used multivariate sparse partial least square discriminant analysis, a supervised learning method implemented in mixOmics v6.8.5 R package, (Le Cao *et al.*, 2016), to identify the signature discriminating the HDGEC from HC group.

Exploratory analyses

Clinical outcomes and correlation with gut microbiome

We used an independent samples *t*-test to examine differences in cognitive performance (HD-CAB composite score) between our HDGEC and HC groups. Next, using only the HDGECs, we examined associations among the gut microbiome, cognitive outcomes (HD-CAB composite score) and clinical outcomes related to Huntington's disease (UHDRS–TMS, UHDRS–TFC and CAP$_S$), using Spearman's rank correlation coefficients, given the unevenness in distribution of our gut variables. For the associations among UHDRS–TMS, UHDRS–TFC and bacterial data, it was necessary to limit analysis to only manifest HDGECs, because these clinical measures, which were developed for use in symptomatic HD, exhibit substantial range restrictions (ceiling effects) in the premanifest group, where motor symptoms and functional decline are not yet readily apparent. Furthermore, we investigated the association between bacterial data and CAP$_S$ scores only for premanifest HDGECs, given that CAP$_S$ is a predictive measure of symptomatic disease onset. Gender and age variables did not significantly correlate with these clinical outcomes.

16S rRNA sequencing produces thousands of gut variables, hence it is essential to restrict analyses of these variables to limit Type 1 error. Thus, we selected bacteria outcomes based on previous research in Parkinson's disease and other conditions, which has documented associations among particular bacteria, disease progression and cognition (Bajaj *et al.*, 2012; Bruce-Keller *et al.*, 2015; Manderino *et al.*, 2017; Parashar and Udayabanu, 2017; Gerhardt and Mohajeri, 2018; Zhan *et al.*, 2018; Weis *et al.*, 2019). For these analyses, at the Phylum level we examined Bacteroidetes, Firmicutes, Verrucomicrobia and Proteobacteria; at the Family level, we examined *Akkermansiacea*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Christensenellaceae*, *Lachnospiraceae*, *Ruminococcaceae* and *Streptococcaceae*. We also examined a single species, *E. hallii*.

Data availability

Raw data from these analyses are available upon reasonable request.

Results

In terms of the *richness* and evenness of the microbial environment, which is typically associated with resilience of the gut environment, HDGECs showed less alpha diversity compared to HCs. Specifically, for alpha diversity, Observed and Fisher indices were lower ($P = 0.001$), indicating fewer species present and lower evenness, in HDGECs than in HCs; Fig. 1A. Beta diversity, indicating microbial community structure, significantly differed in HDGECs compared to HCs, based on unweighted UniFrac distances, $P = 0.001$; Fig. 1B.

Next, using analysis of composition of microbiomes to examine differences observed in gut microbiome composition at the Phylum and Family levels, we found a significant gender by group interaction, with several differences identified in males. Specifically, for males, at the Phylum level, Euryarchaeota, Firmicutes and Verrucomicrobia differed significantly between groups. At the Family level, many Families differed significantly by gender between groups, including *Acidaminococcaceae*, *Akkermansiaceae*, *Bacteroidaceae*, *Bifidobacteriaceae*, *Christensenellaceae*, *Clostridiaceae*, *Coriobacteriaceae*, *Eggerthellaceae*, *Enterobacteriaceae*, *Erysipelotrichaceae*, *Flavobacteriaceae*, *Lachnospiraceae*, *Methanobacteriaceae*, *Peptococcaceae*, *Peptostreptococcaceae* and *Rikenellaceae* for males. For females, neither the Phylum nor the Family level revealed significant differences.

In terms of functional gut pathways, Kyoto Encyclopedia of Genes and Genomes Ortholog abundances differed significantly between HDGECs and HCs in both alpha and beta diversity. Specifically, for alpha diversity, the HDGEC group showed significantly less richness and evenness in functional pathways (Observed and Fisher) compared to HCs ($P = 0.001$). HDGECs and HCs also differed significantly in composition of gut pathways (beta diversity, $P = 0.001$). Additional analyses on predicted pathways using sparse partial least square discriminant analysis revealed specific functional gut pathways affected in the HDGEC group, including the Super pathway of serine and glycine biosynthesis, Starch degradation V pathway, Methylerythritol phosphate pathway I, Methylerythritol phosphate pathway II and the NAD biosynthesis I pathway (Fig. 2). At the enzyme level, enzymes of glutathione transferase and homoserine O-succinyltransferase were significantly different in the HDGEC group compared to HCs (Fig. 3).

Although the samples within the HDGEC group were small, we explored whether the manifest HDGECs ($n = 19$) differed from the premanifest HDGECs ($n = 23$). We found no statistically significant differences in richness, evenness and in group microbial composition in the gut, from alpha and beta diversity measures. Differences describing microbial composition, as measured by taxonomic analysis, also showed no differences between these groups, apart from a significant difference in the abundance of *Ruminococcaceae* family, for male HDGECs only. Given these findings, we limited our examination of the gut results presented above, to comparisons between the combined HDGEC group (manifest and premanifest HD) and HCs.

Interestingly, in our exploratory analyses linking the gut measures with clinically relevant characteristics in the HDGECs, we found several associations, see Fig. 4. In particular, in manifest HDGECs, lower abundance of *E. hallii* correlated with more severe motor signs (UHDRS–TMS; $r_s = -0.512$, $n = 16$, $P = 0.043$), however, we did not find associations between *E. hallii* abundance and our clinical measure of functional capacity (UHDRS–TFC; $r_s = 0.329$, $n = 19$, $P = 0.183$). In premanifest HDGECs, we observed a significant negative relationship between the abundance of *E. hallii* and estimated proximity to disease onset (CAP$_S$), $r_s = -0.568$, $n = 21$, $P = 0.007$.

Prior to undertaking an evaluation of the cognitive measures in the context of the gut outcomes, we examined group differences in overall cognition to establish that as expected, the HDGEC group was cognitively impaired compared to the HC group. Group analysis confirmed this expected difference. Overall, the HDGEC group performed more poorly than controls on the HD-CAB composite measure of overall function [$t(76) = 3.940$, $P < 0.001$], with a large effect size (Cohen's $d = 0.91$), see Fig. 5.

In terms of associations between cognition and gut outcomes, *E. hallii* was associated with memory performance on the Hopkins Verbal Learning Test-Revised, $r_s = 0.315$, $n = 42$, $P = 0.042$, and a similar trend was observed for a measure of processing speed and attention, Symbol Digit Modalities Test, $r_s = 0.278$, $n = 42$, $P = 0.074$. These associations, however, appeared in the context of many other non-significant correlations, and
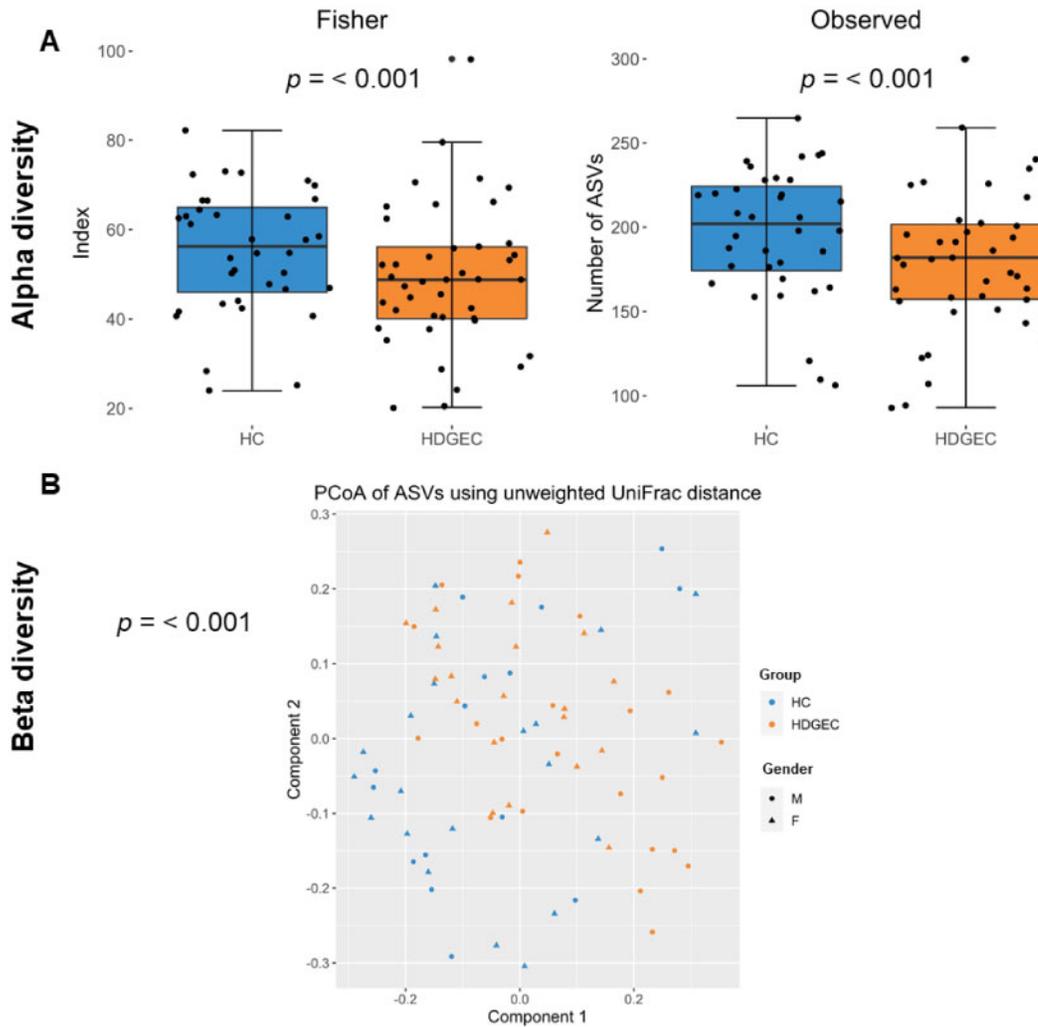
Figure 1 Alpha and beta diversity differences in the gut microbiome in HDGEC and HC groups. (**A**) Alpha diversity analysis investigating richness (Observed) and evenness (Fisher) in HDGEC versus HC groups. (**B**) Beta diversity analysis showing variation in the composition of the samples based on their phylogenetic distance (unweighted UniFrac distance) in HDGEC versus HC groups.
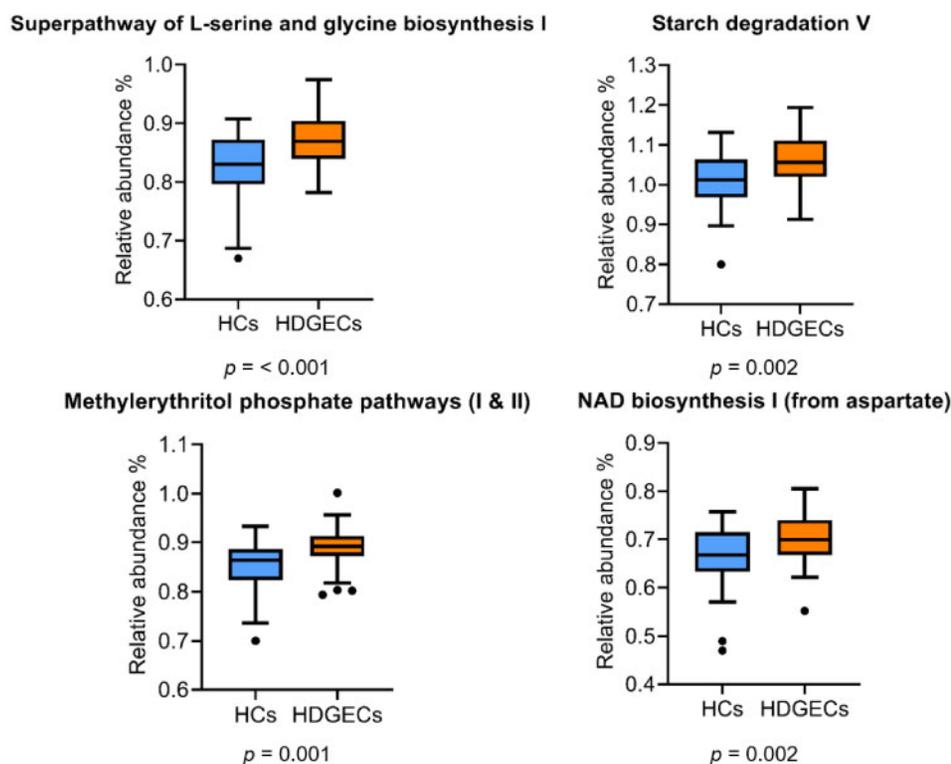
therefore must be interpreted with caution as they may be spurious. Supplementary Tables 2–4 show the full extent of these correlational analyses among gut bacteria data, clinical measures and cognitive performance in HDGECs. Supplementary Table 5 details correlations between self-reported gastrointestinal symptoms (Gastrointestinal Health Appraisal Questionnaire) and alpha diversity indices, which were non-significant.

Discussion

The rationale for examining the gut in Huntington's disease is supported by several lines of evidence linking differences in gut microbiota changes across a variety of diseases (Dinan and Cryan, 2017; Kowalski and Mulak, 2019; Sarkar and Banerjee, 2019), clinical indicators of gut dysfunction in Huntington's disease (e.g. weight loss, nutrition intake abnormalities and gastrointestinal issues), and recent evidence of gut dysfunction in Huntington's disease mice (Kong *et al.*, 2018). Using 16S rRNA gene sequencing, our study provides the first evidence of gut dysbiosis in people with Huntington's disease. These results supported our primary hypothesis of an altered gut microbiome in HDGECs compared to age- and gender-matched HCs, in terms of differences in richness, evenness and overall microbial composition of the gut, as indicated by alpha and beta diversity, and further described by taxonomic changes and functional pathway and enzyme differences. We also discovered consistent trends among Huntington's disease progression, gut microbiota and cognitive outcomes, although these must

Functional pathway differences in the gut microbiome between HDGECs and HCs

Superpathway of L-serine and glycine biosynthesis I

Starch degradation V

Methylerythritol phosphate pathways (I & II)

NAD biosynthesis I (from aspartate)

Figure 2 Functional pathways differences in the gut microbiome between HDGEC and HC groups.

Enzyme differences in the gut microbiome between HDGECs and HCs

Glutathione transferase
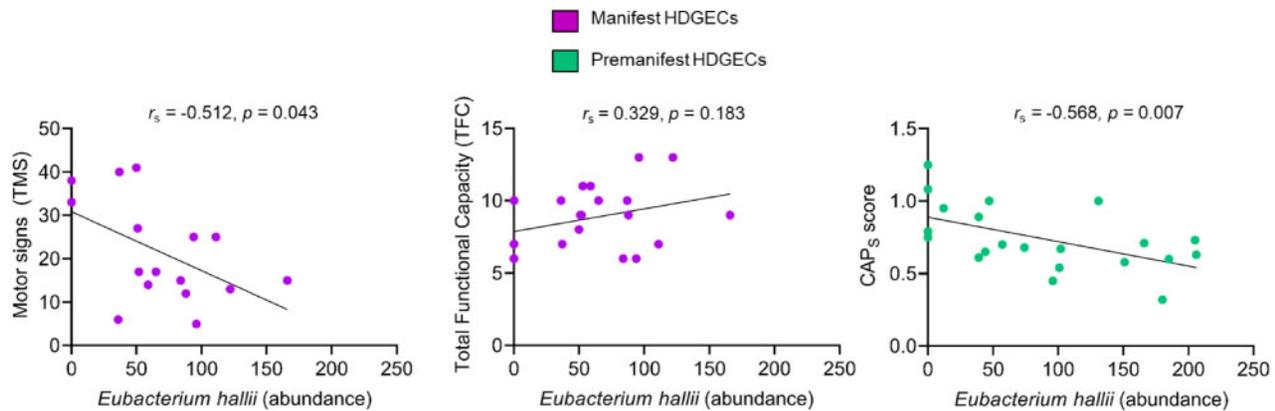
Homoserine O-succinyltransferase
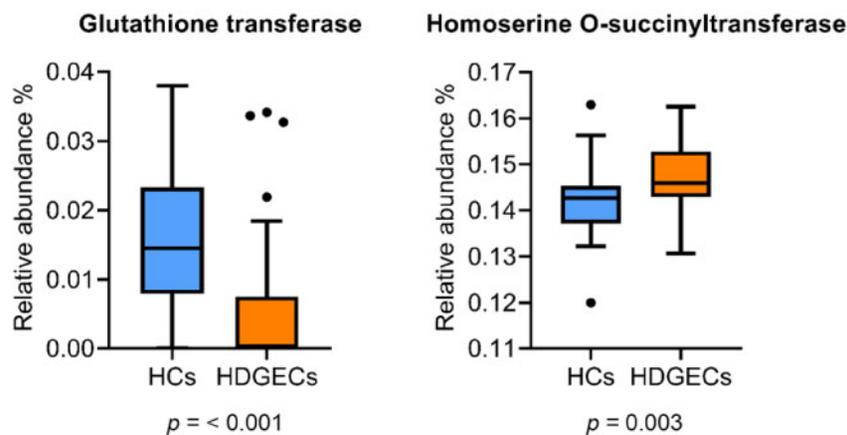
Figure 3 Enzyme differences in the gut microbiome between HDGEC and HC groups.

be interpreted with caution given our small sample size and the need to conduct several statistical tests.

Consistent with our current findings in Huntington's disease of lower alpha diversity and beta diversity differences, indicating less than normal richness, evenness and altered microbial gut composition, others have reported similar observations in neurodegenerative conditions such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Keshavarzian *et al.*, 2015; Rowin *et al.*, 2017; Vogt *et al.*, 2017). Whilst the bacteria

Figure 4 Relationship among *E. hallii*, clinical measures of disease progression in manifest HDGECs (TMS & TFC) and estimated disease onset (CAP$_S$) in premanifest HDGECs.
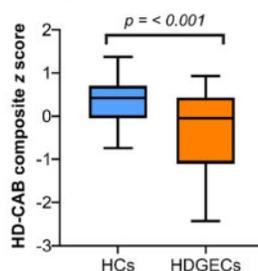
Figure 5 Overall performance between groups (HDGEC and HC) on cognitive outcomes (HD-CAB composite score).

responsible for observed changes in diversity metrics vary across the spectrum of diseases, altered diversity of the gut microbiota across these diseases indicates that gut dysbiosis appears to be a common feature of at least some neurodegenerative diseases.

Homeostasis of the gut microbiome is important for human health, and influences behaviour and brain function via the gut–brain axis (Dinan and Cryan, 2017). Thus, some of the clinical manifestations or brain changes evident in Huntington's disease may be related to gut-driven modulation of brain inflammatory pathways, via communication among the gut, endocrine, immune and neural pathways (Burokas *et al.*, 2015). Studies in Alzheimer's disease and Parkinson's disease support a link between the gut and neuropathology, with evidence that microbiota influence amyloid development in Alzheimer's disease (Kowalski and Mulak, 2019), and α-synuclein pathology in Parkinson's disease (Sampson *et al.*, 2016; Fitzgerald *et al.*, 2019). Further studies of Huntington's disease will be needed to determine

associations between the gut microbiota and Huntington's disease-specific neuropathology.

Although we observed differences between HDGECs and HCs at the taxonomy level, including gender effects, we did not observe an increase in Bacteroidetes and a proportional decrease in Firmicutes, such as that reported in the preclinical Huntington's disease study by Kong *et al.* (2018). In our sample, the differences observed in Phyla and Families had some, but not complete overlap with published results from other neurodegenerative diseases, which is not surprising given the discrepancy in the literature both amongst and between different clinical populations (for a full review see: Gerhardt and Mohajeri, 2018). Our finding of significantly lower abundances of Firmicutes, *Lachnospiraceae* and *Akkermansiaceae* in HDGEC males is interesting, because it links to inflammatory processes, given the role of *Akkermansiaceae* in the maintenance of the gut barrier (Chelakkot *et al.*, 2018), and *Lachnospiraceaes*' and Firmicutes' role in the production of butyric acid, reducing inflammation (Louis *et al.*, 2010; Stilling *et al.*, 2016; Truax *et al.*, 2018). Previously, reductions in these bacteria have been shown to be related to depression (Huang *et al.*, 2018), but we found no such significant associations when controlling for depressive symptoms or age. The finding of a gender effect from taxonomic analyses indicates that the gut is affected not only by Huntington's disease, but also by other factors such as sex, which could interact with the relationship between gut microbiota and disease progression. Whilst differences in gut composition are interesting, particularly in the early phase of our understanding of the gut microbiome in conditions such as Huntington's disease, ongoing research must determine whether observed changes in the relative abundances of bacteria are a cause, or a product of abnormal gut–brain function.

Differences in gut composition in Huntington's disease compared to HCs do not necessarily translate into gastrointestinal problems. In contrast, differences in gut function have, by definition, consequences that may be clinically relevant. For example, the functional pathway and enzyme differences observed in our study related to serine, methionine (including enzyme differences in homoserine) and glycine biosynthesis. These changes may be important, because methionine links to oxidative stress (Campbell *et al.*, 2016), and these pathways are related to glutamate release, tryptophan metabolism and synthesis of serotonin and melatonin production (O'Mahony *et al.*, 2015; Yu and Lau, 2018; Hoglund *et al.*, 2019). The finding of differences in these pathways in our Huntington's disease sample supports previous observations showing abnormal handling of tryptophan metabolism in Huntington's disease, resulting in higher levels of oxidative stress, which may contribute to neuroinflammation and brain dysfunction, as well as depressive symptoms and memory difficulties (Stoy *et al.*, 2005; Mendelsohn *et al.*, 2009; Bourassa *et al.*, 2016; Kałużna-Czaplińska *et al.*, 2019; Gao *et al.*, 2020). Furthermore, at the enzyme level, significantly lower levels of glutathione transferase have been found in Parkinson's disease and Alzheimer's disease, and these changes are linked to increased oxidative stress and neuroinflammation (Mazzetti *et al.*, 2015). Thus, our finding in the HDGEC group of functional pathway and enzyme differences raises interesting questions about the possibility of future development of gut biomarkers.

Published evidence relates cognition to bacteria across a range of taxonomic levels, but previous studies in other neurodegenerative samples have yielded mixed findings of relative higher or lower abundance of specific bacteria and their associations with cognition and disease progression (Keshavarzian *et al.*, 2015; Vogt *et al.*, 2017; Fitzgerald *et al.*, 2019; Sarkar and Banerjee, 2019). Although speculative, the consistent trend we observed among *E. hallii*, Huntington's disease progression and cognition is also interesting given this bacterium's role in short-chain fatty acid production, tryptophan metabolism and inflammation. We did not see any association among Verrucomicrobia, Bacteroidetes, Proteobacteria and cognitive outcomes, which has been reported in other studies such as Manderino *et al.* (2017).

This study provides the first evidence of gut dysbiosis in people with Huntington's disease, adding to the growing number of studies that have demonstrated an altered gut microbiome profile in different neurological, and particularly neurodegenerative populations. Understanding the interplay among the gut–brain axis, gut microbiome and the pathogenesis of disease progression remains an ongoing challenge, particularly given the large number of variables to analyse, and the inherent challenge of establishing a large enough sample size in humans to reliably establish meaningful differences. This is particularly relevant in studies such as ours, which are limited by small sample sizes, a cross-sectional design, and other confounding variables that make assessing healthy gut microbiota challenging, given the influence of many factors such as age, medication, diet, drugs, environment and lifestyle factors. Nonetheless, from a Huntington's disease perspective, given the clinical evidence of gut symptoms in Huntington's disease, recent evidence of an altered gut microbiome in Huntington's disease mice (Kong *et al.*, 2018), and now our study describing altered gut microbiota in humans with Huntington's disease, these initial results highlight the importance of understanding how the gut fits into the progression of Huntington's disease. To fully understand this key aspect of the disease, larger and longitudinal studies are essential. Furthermore, our results raise the prospect of identifying gut biomarkers in Huntington's disease, which will be enabled by further examination and characterization of the gut microbiome in relation to other phenotypic and possibly genotypic information. Lastly, these results raise the tantalizing proposition of whether the gut may be a potential target for future therapeutic intervention to improve outcomes in Huntington's disease and other neurodegenerative diseases.

Supplementary material

Supplementary material is available at *Brain Communications* online.

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Competing interests

J.S. is director of a research company, Zindametrix, which supports clinical trial sponsors in the use of the HD-CAB and other cognitive testing in clinical trials, and provides consulting services regarding cognitive assessment in Huntington's disease to clinical trial sponsors via Stout

Neuropsych Pty Ltd. The remaining authors have no competing interests to report.

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