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UNIVERSITY OF CALIFORNIA SAN DIEGO

Salivary Huntingtin in Huntington's Disease

A Thesis submitted in partial satisfaction of the requirements
for the degree Master of Science

in

Biology

by

Ameera Samaher Haque

Committee in Charge:

Jody Corey-Bloom, Chair
Shelley Halpain, Co-Chair
Ella Tour

2018

The Thesis of Ameera Samaher Haque is approved and it is acceptable in quality and form for publication on microfilm and electronically:

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Co-Chair

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Chair

University of California San Diego

2018

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ACKNOWLEDGEMENTS

Those of us lucky enough to know Dr. Jody Corey-Bloom know her passion for her work and for people, particularly her patients. Working with you, I have been fortunate to observe first-hand the importance of clinical research and, more importantly, of hope in medicine. In addition to neuroscience, her advice and guidance over the years have taught me life skills that cannot be from a textbook. Her incredible work ethic and empathy is enviable, and I have been lucky to learn from her for the past few years. Thank you for your guidance and mentorship, I am eternally grateful.

I would also like to thank Dr. Elizabeth Thomas, our vivacious collaborator, without whom this project would not have been possible. Thank you for the countless hours you spent in lab assaying samples, I truly appreciate all your hard work.

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ABSTRACT OF THE THESIS

Salivary Huntingtin in Huntington's Disease

by

Ameera Samaher Haque

Master of Science in Biology

University of California San Diego, 2018

Professor Jody Corey-Bloom, Chair
Professor Shelley Halpain, Co-Chair

Huntington's disease (HD), is an autosomal dominant disorder caused by an expansion of the CAG sequence in the huntingtin gene, which encodes the ubiquitously expressed Huntingtin (Htt) protein. Diminished motor function, cognition, and functional capacity are common symptoms. HD affects 10 in every 100,000 individuals in the US, with symptoms showing substantial variability in age at onset, severity and course of illness; thus warranting the need for reliable biomarkers to anticipate the onset of the disease and track its progression. The protein associated with the disease, huntingtin (Htt), is released into the extracellular fluid as neurodegeneration occurs in HD, and is thus a potentially useful biomarker. It has been

previously measured in CSF and blood plasma in HD; however, these are invasive techniques with varying yields. We therefore wondered if Htt protein could be reliably measured in saliva.

We assayed 98 saliva samples from manifest(HD), premanifest(PM), and age-matched normal control(NC) participants and found that salivary total Htt (tHtt) was significantly increased in HD saliva compared to NC. Salivary tHtt showed no gender effects and was significantly positively correlated with age in HD. As expected, tHtt levels had a positive correlation with motor symptoms and a negative correlation with cognitive ability and functional capacity in HD. Thus, tHtt protein can be reliably measured in human saliva and shows promise as a non-invasive clinically meaningful biomarker of disease progression in HD.

INTRODUCTION

Huntington's disease (HD) is a rare autosomal dominant disorder caused by the inheritance of a polymorphic polyglutamine mutation in exon 1 of the *HTT* gene that encodes for huntingtin (Htt) protein (Saudou et al, 2016). It affects approximately 10 individuals per 100,000 in the western population, but many more are at risk for the disease since there is a 50% chance of inheriting the mutation from a parent (Tabrizi et al, 2009). The clinical diagnosis of HD is based upon the manifestation of motor impairment symptoms, primarily the onset of chorea or choreic movements, a hallmark symptom of the HD phenotype. The mean age of disease onset occurs at mid-forties; however, this does not account for cases in which behavioral, cognitive, or psychiatric symptoms precede motor symptoms. As such, although the genotype of the disease is known, there is still a lack of information on both the molecular basis and the clinical characteristics of disease progression. Currently in the field of Huntington's Disease, there are no biomarkers accepted that can predict disease onset or track disease progression.

The wild type *HTT* gene has a polyglutamine stretch on the N-terminal that ranges from 9 to 35 repeats of the CAG codon, with an average of 17-20 CAG repeats for the normal genotype (Saudou et al, 2016). Wild type huntingtin (Htt) protein is ubiquitously expressed and is necessary for life. The knockout of wild type Htt protein is fatal in mice—leading to death at mouse embryonic day 7.5 (Saudou et al, 2016). Htt is critical for forebrain development, and deletion of a functional *HTT* allele compromises the viability of neurons in that region (Reiner et al, 2003). Additionally, normal huntingtin is involved in several immune cell processes including immune cell migration and apoptosis (Ramdzan et al, 2017; Kwan et al, 2012).

In contrast, the mutant *HTT* gene produces mutant huntingtin (mHtt) protein that has an abnormal polyglutamine expansion on the N-terminus. People with 36 or more repeats of the

CAG codon are considered "gene positive" for HD (Tabrizi et al, 2009). In other words, if an individual has a CAG repeat number 36 and greater, he or she has the gene for Huntington's disease, and has a 50% chance of passing it on to their offspring – an inheritance pattern that is expected of an autosomal dominant disorder. Gene positive individuals with no clinical symptoms of HD are characterized as premanifest (PM), while those who have started to show symptoms are categorized as manifest HD. Although the disease is fully penetrant, there is an age-dependent penetrance pattern shown if CAG repeat lengths range between 36 and 39 (Ross, 2014). Individuals within this range of CAG repeats are still gene positive for HD but may show symptoms later in life relative to those with CAG repeats of 40 and greater (Ross et al, 2014).

Intracellularly, mutant Huntingtin (mHtt) protein is cleaved by proteases and the abnormal N-terminal fragments either translocate to the nucleus, which interrupts transcriptional activity, or aggregate in the cytosol, which impedes normal cellular processes (Saudou et al, 2016). In both cases, the result is neuronal dysfunction and ultimately death (Saudou et al, 2016). Analysis of brain magnetic resonance imaging (MRI) of 123 normal control, 120 premanifest HD, and 123 manifest HD individuals showed that the regions of greatest neurodegeneration in HD were the striatum, caudate nuclei, and putamen. All three structures were significantly smaller, by percentage of intracranial volume, in both premanifest and manifest HD subjects compared to normal controls (Tabrizi et al, 2009). As these neurons die, they release all of their cellular contents, including the Huntingtin protein fragments and aggregates, into the extracellular environment. Thus, measuring levels of Htt protein could reflect neurodegeneration in HD.

In recent years, new genetic therapies have been proposed — the most promising of which seeks to lower total Huntingtin (tHtt) protein levels, mutant and wild type, using an

antisense oligonucleotide (ASO) against the mRNA of the *HTT* gene (Wild et al, 2014). This drug is designed to prevent further translation of the *HTT* mRNA into the Htt protein, thus lowering the overall amount of both mHtt and wild type Htt protein, which should theoretically decrease neuronal death (Roche Press Release, 2017). As such, the ability to measure and monitor Htt protein levels has become even more crucial. Changes in either tHtt or mHtt levels could then also provide an indication of the efficacy of the gene silencing therapies.

Cerebrospinal fluid (CSF) has been a highly desirable biosample to use in such assays, since 20% of CSF protein is known to have originated from the brain (Byrne and Wild, 2016). Thus, as neurodegeneration progresses, Htt protein released from dying neurons should be detectable in CSF from which it can be quantified (Byrne, Wild, 2016). A prior study was successful in detecting total Huntingtin protein (Htt and mHtt) in plasma and CSF using a highly-specialized time-resolved Forster resonance energy transfer (FRET) assay (Weiss et al, 2009). However, total Htt yields were quite low and no significant relationship could be established between tHtt protein levels and disease progression.

Currently, one of the biggest challenges is the inability to recreate promising findings from previous studies in larger cohorts. The FRET assay is also quite expensive, and resource constraints have further prevented larger follow up studies. Additionally, bio-specimens that are compatible with the available assays require highly specialized facilities, materials, and staff to collect, which complicates frequent specimen collection. CSF must be collected by lumbar puncture which requires a neurologist, anesthesiologist, or similarly trained personnel, while plasma must be collected via blood draw which requires a phlebotomist. Also, both procedures can only be conducted in designated medical facilities and the process can be quite daunting for

patients. All in all, these factors hinder recruitment for such ambitious projects and create yet another obstacle in this endeavor.

Saliva has many advantages as a diagnostic fluid as it can be collected non-invasively by individuals with minimal training, and there is little to no risk of contracting infection during collection. Importantly, several proteins related to other neurodegenerative diseases have been measured in saliva such as amyloid beta (Lee, 2017) and tau (Shi, 2011) in Alzheimer's disease, and alpha-synuclein (Vivacqua, 2016) and DJ-1 (Devic, 2011; Masters, 2015) in Parkinson's disease. Saliva is a very dilute liquid comprised of approximately 99% water but contains several electrolytes, enzymes, mucins, and proteins that either originate from the blood or are secreted by the salivary glands (Humphrey, 2001). Unaided passive diffusion is the most common route from blood to saliva. Capillaries surrounding the salivary glands are porous and allow biomolecules from blood to diffuse into saliva (Pfaffe, 2011). These constituents must pass through five barriers to enter the saliva: (1) the capillary wall, (2) the interstitial space, (3) the basal cell membrane of the salivary gland cell, (4) through the cytoplasm of the salivary gland cell, (5) and finally through the luminal cell membrane of the salivary gland cell and into the saliva (Fig. 1), as such, smaller nonpolar molecules are more likely to diffuse. Molecules can

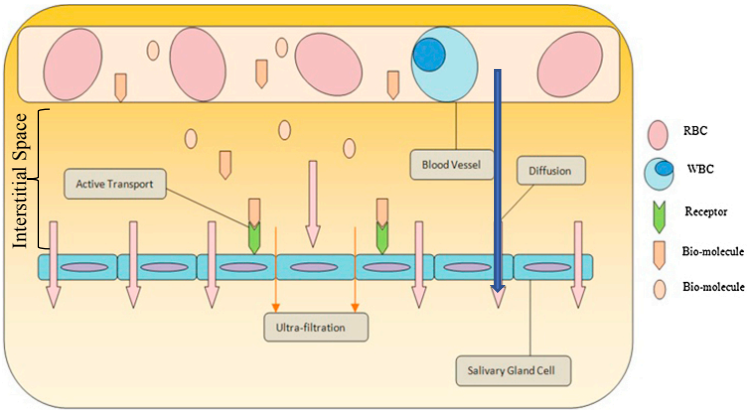

Figure 1: Schematic diagram illustrating routes by which molecules enter saliva from blood.

also enter the saliva via active transport, through the secretory cells of the glands, or via

ultrafiltration, which transports molecules from the blood into the saliva through the gaps between salivary gland cells (Fig. 1).

As previously discussed, changes in huntingtin protein levels can indicate neurodegeneration, a hallmark of HD. The ability to track neurodegeneration safely and reliably is of utmost importance in HD. Additionally, reliable quantification of tHtt protein is particularly timely since tHtt protein levels can serve as a marker of efficacy in Htt-lowering gene therapy studies. This could be a potential breakthrough for non-invasive specimen collection for HD patients, as the saliva collection process requires no specialized equipment, only minimal training to conduct, and most patients are able to provide a sample with little to no difficulty. In this project, we sought to determine first whether Htt protein could be identified and reliably measured in human saliva; next, if salivary levels of tHtt are clinically meaningful, i.e. do they distinguish PM and manifest HD subjects from NC and do they correlate with clinical measures .

METHODS

Participants

This study was conducted in accordance with the requirements of the Code of Federal Regulations on the Protection of Human Subjects and was approved by the University of California, San Diego Institutional Review Board. Informed consent was obtained from all subjects prior to their participation. Patients were recruited through the University of California, San Diego (UCSD) HD Clinical Research Center. Premanifest and manifest HD participants were recruited based on a CAG repeat length of 36 or more. Manifest HD required a definitive diagnosis of HD. Normal controls were screened for reported history of neurological or psychiatric disorders or any use of psychoactive substances. All participants provided demographic information such as sex, age, and education, and a thorough medical and family history including parental age of onset, age of onset, comorbid conditions, and medication status.

Sample Collection & Processing

Saliva

All saliva samples were collected via the passive drool method. Participants were asked to refrain from smoking, eating, drinking, or oral hygiene procedures for at least 1 hour prior to the visit. Next, participants thoroughly rinsed their mouths with water to clean any residual contamination and waited for a 30-minute period to allow saliva to reconstitute prior to collection. Participants were asked to imagine they are chewing their favorite food and guide the pooling saliva into a collection vial. Larger tubes were used for participants manifesting more HD symptoms who have difficulty coordinating the movements required to guide the saliva into

the tube. Approximately 2 milliliters of unstimulated whole saliva were collected from each participant. These samples were aliquoted into 500 microliter fractions by pipetting, and immediately stored at -80C until assaying. At the time of use, saliva fractions were thawed and centrifuged at 10,000 g for 10 minutes at 4C to remove any insoluble components and cellular debris. The supernatants were collected and used for all assays.

Plasma

All blood draws were conducted by a licensed phlebotomist. Whole blood was collected in K_2EDTA coated BD Vacutainers, and immediately centrifuged at 10,000 g for 10 minutes at room temperature. The separated blood plasma layer was aliquoted into 500 microliter fractions and stored at -80C. At the time of use, plasma aliquots were thawed and used for all assays in the laboratory of Dr. Elizabeth Thomas at the Scripps Research Institute.

Western Blotting

Human saliva supernatants were concentrated 4-fold by vacuum centrifugation or used un-concentrated (1X; designated as "neat"). 18 µl of concentrated or neat saliva was loaded into each well. Samples were separated by 7% SDS-PAGE and blotted onto nitrocellulose membranes using standard procedures as described previously, Ponceau's stain was used to verify the transfer of proteins to the membrane. For immunodetection, the mouse anti-Htt antibody MAB2166 (Millipore;1:2,000 dilution) was used, followed by a goat anti-mouse HRP-conjugated secondary antibody (Pierce; 1:2,000 dilution). Immunoreactive bands were visualized using Pierce enhanced chemiluminescence (ECL) Western Blotting Substrate (Pierce). Gel images were acquired using a Fluorochem E Imager. All Western blot analysis was conducted by Dr. Elizabeth Thomas.

ELISA Measurements

Total Htt protein levels in saliva samples were quantified using a commercially available ELISA kit (LifeSpan BioSciences, Inc.) according to the manufacturer's protocol using 50 µl of saliva per well diluted 1:1 with the provided Sample Dilution Buffer. Operators performing the assays were blinded to the clinical state of the participant. For the plasma samples, 50 µl of plasma was diluted 1:2 with Sample Dilution Buffer. The recombinant Htt protein standard corresponded to amino acids 802-940 of the human Htt protein, with antibodies corresponding to protein fragments including this region. The accuracy and precision of this ELISA in saliva was assessed by testing the recovery of a spiked-in control and the linearity of dilution of the spiked in control in two independent saliva samples. We found that the spiked-in recovery for the saliva matrix was 91.2% +/- 4.05% and the R^2 value for linear regression = 0.976. Samples were assayed for cortisol using an immunoassay kit optimized for saliva (Salimetrics, LLC in Carlsbad, CA) following the manufacturer's recommended protocol. All assays were performed by Dr. Elizabeth Thomas.

Clinical Assessments

As typical symptoms of Huntington's disease include motor abnormalities, cognitive difficulties, and psychiatric issues, the following clinical assessments were conducted on all the manifest HD, premanifest HD, and normal control participants in this study to evaluate the clinical state of each individual.

Motor Assessments

Dr. Corey-Bloom administered the Unified Huntington's Disease Rating Scale Motor Assessment which assessed all participants for motor abnormalities in eye movement such as

ocular pursuit, saccade initiation, saccade velocity; oral motor abnormalities such as dysarthria or slurred speech, tongue protrusion; motor abnormalities in the upper extremities such as finger taps, hand pronation/supination, arm rigidity or stiffness; body bradykinesia or slowing; maximal dystonia, involuntary muscle contractions that cause repetitive or twisting motions; maximal chorea, involuntary jerky dance-like movements that are the hallmark symptom of Huntington's disease; and abnormalities of gait and balance. Each category is scored from 0 to 4; 0 representing normal function and 4 indicating the most severe dysfunction. Ocular pursuit, saccade initiation, and saccade velocity were assessed in both the horizontal and vertical directions. Finger taps, hand pronation/supination, and arm rigidity for the left and right side were assessed separately. Finally, both maximal dystonia and were evaluated in five regions of the body: trunk, right upper extremity, left upper extremity, right lower extremity, and left lower extremity. Maximal chorea was also evaluated in the face and the buccal oral lingual region. The maximum score possible is 124 and higher scores indicate greater motor symptom manifestation. The total sum of points on this assessment comprises the Total Motor Score (TMS) in this experiment, while the sum of all maximal chorea sub-scores, maximum score of 28 points, is shown as the Total Chorea Score (TCS).

The final motor assessment used in this battery was the Timed Up & Go test. This test measures the amount of time (in seconds) a participant takes to walk to a point located 10 feet away, turn, and return to the starting position. Manifest HD subjects with more balance and gait problems are expected to take longer to complete this task compared to normal controls.

Cognitive Assessments

All subjects were assessed using three separate cognitive assessments: the Mini-Mental State Exam (MMSE), the Montreal Cognitive Assessment (MoCA), and the Symbol Digit

Modalities test (SDM). The MMSE tests five areas of cognitive function: orientation, registration, attention, calculation, recall, and language. Each correct answer adds one point to the score, for a maximum possible total of 30 points. The MoCA tests orientation, memory and recall, delayed recall, language, abstraction, attention, and visuospatial abilities, also for a maximum possible total of 30 points. The SDM measures information processing speed and efficiency using a symbol/digit substitution task. Participants are provided with a key of unique symbols paired with digits 0-9. The rows below contain only symbols, and s/he is required to write the correct number, corresponding to the symbol, in the designated space below the symbol. This test is timed for 90 seconds and the maximum possible score is 110.

Functional and Behavioral Assessments

All subjects were assessed using the Unified Huntington's Disease Rating Scale for Total Functional Capacity. This scale assesses the participant's care environment and his/her capacity to (1) engage satisfactorily in paid or voluntary work, (2) engage in personal and family finances, (3) carry out routine domestic tasks, and (4) complete activities of daily living (ADL) such as dressing, bathing, and eating. The maximum possible score is 13, indicating that the participant has the capacity to perform all the tasks specified independently and can care for themselves. Any loss of capacity to execute these tasks results in points lost from the 13.

All subjects were also assessed using two behavioral scales: the self-reported Hospital Anxiety and Depression Scale and the Snaith Irritability Scale (HADS-SIS), and the rater evaluated Problem Behaviors Assessment short form (PBA-s). The HADS-SIS assesses anxiety, depression, and irritability based on self-reported responses on a 22-point patient questionnaire. The maximum possible score is 66, once again higher scores indicate more behavioral issues. The PBA-s evaluates the severity and frequency of 11 items: depressed mood, suicidal ideation,

anxiety, irritability, angry or aggressive behavior, lack of initiative (apathy), perseverative thinking or behavior, obsessive-compulsive behavior, delusions or paranoid thinking, and hallucinations. Each item is rated on a 5-point scale from 0, indicating the absence of the trait, to 4, the most severe and frequent occurrence, by a certified rater. The maximum possible score is 160, with higher scores indicating greater manifestation of behavioral symptoms.

Disease Burden Scores

Disease burden scores have been proposed in the field of HD clinical research in an attempt to quantify the extent of disease progression for each subject at a particular point in time (Harrington, 2015). They can also be interpreted as measures of cumulative genetic toxicity also referred to as genetic burden; supposedly, higher scores indicate greater disease burden than lower scores. Although there is not enough reproducible data to establish strict endpoints distinguishing premanifest from manifest individuals, disease burden scores are typically used in clinical HD research to stratify premanifest patients as either far from or close to phenoconversion. Disease burden scores typically take into account the age of the subject and their CAG repeat. The CAP score (Zhang, 2011), which is computed as CAP = Age_0 X (CAG-33.7), where Age_0 is the age at the time of the study visit, is the disease burden score that we have chosen to use for this study.

Statistics

All statistical analyses were run using IBM SPSS and graphs were generated using Prism GraphPad 7. Outliers were assessed from the raw data set using the Grubb's outlier test. The study consisted of participants from 3 diagnostic groups: HD gene positive participants were clinically classified as manifest HD or premanifest HD, and normal controls. Premanifest HD participants were significantly younger than participants in the other diagnostic categories, as determined by one-way ANOVA, thus all further calculations were controlled for age to account for this. The distribution of the data values in each diagnostic group was tested for normality using the Kolmogorov-Smirnov normality test. Difference between mean tHtt levels in each diagnostic group was assessed by age controlled ANCOVA on IBM SPSS. Spearman's r correlations were calculated to consider any relationship between tHtt levels and clinical measures which were not normally distributed.

For certain sub-analyses, HD and premanifest HD participants. were stratified using CAP score > 425 in an effort to capture those HD individuals with greatest disease burden and those premanifest individuals closest to phenoconversion.

RESULTS

Htt protein is present in saliva. We first performed a qualitative western blot using the MAB2166 anti-Htt antibody to determine if total Htt protein could be identified in human saliva. A single band above the 250 kDa marker corresponds to the full-length Htt protein (~350 kDa) in unconcentrated (1x) saliva samples from controls (n=4 in Fig. 2A, and n=4 additional samples in Fig. S1A). In samples that had been concentrated four-fold (4x), this signal increased approximately proportionally (Figs 2A, S1A). Similarly, Htt immunoreactivity corresponding to the full-length protein was seen in 3 out of 4 of the concentrated (4x) saliva samples from HD patients (Fig. 2B), however, levels were not readily detected in unconcentrated (1x) samples (Fig. 2B). We could detect the Htt protein, using a different anti-Htt antibody, 4E10, in HD patient samples, but not controls (Fig. S1B).

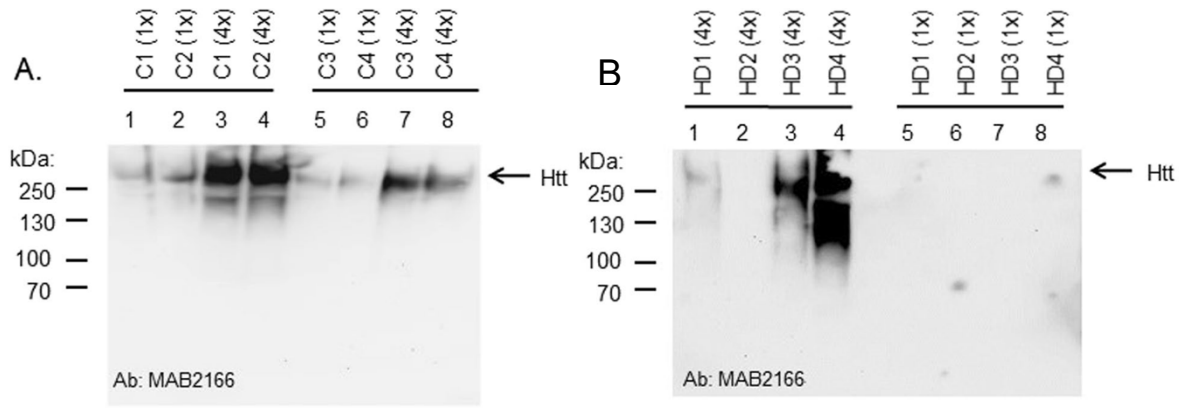

Figure 2: Western blot of salivary total Htt(tHtt) protein. (A) Samples from normal individuals(C1-C4). Three milliliters unstimulated whole saliva was centrifuged, and supernatants were used as-is or were concentrated 4-fold by vacuum centrifugation. Samples were separated by 7% SDS-PAGE and blotted onto nitrocellulose membranes for immunodetection using MAB2166 antibody, a monoclonal anti-huntingtin antibody which recognizes a fragment of the protein (amino acids 181–810), located outside the polyglutamine region. Gel image was acquired using a Fluorochem E imager. (B) Western blot of Htt protein in saliva from HD patients(HD1-HD4) carried out as in (A).

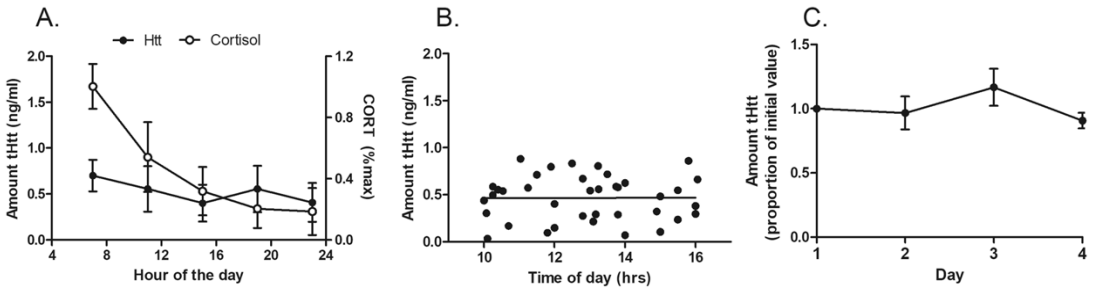

Figure 3: Diurnal stability and reproducibility of salivary tHtt levels. (A) Levels of tHtt from normal controls(n=5) were measured using ELISA at different time points over the course of the day and show no diurnal variation (mean +/- SEM). For comparison, cortisol levels were measured using immunoassays from the same individuals at the same time points. The amount of tHtt reported is relative to a recombinant Htt protein standard corresponding to amino acids 802–940 of the human Htt protein. (B) Salivary levels of tHtt do not vary over the time of sample collection (10 am to 4 pm) in HD patients (n=40; mean+/- SEM).(C) Salivary tHtt is reproducibly detected on different days. Data shows tHtt measures taken on four non-consecutive days from the same individuals (n = 8).

Table 1: Demographic characteristics of diagnostic groups Mean ± SEM [Range]

	NC N = 37	**PM** N = 27	**HD** N = 34	**P-Value** ANOVA
Age (Years)	55.22 ± 2.10 [28 - 77]	44.41 ± 2.41^ [28 - 71]	55.06 ± 2.07 [30 - 76]	**0.012**
Education (Years)	14.88 ± 0.46 [12 - 22]	16.29 ± 0.63 [12 - 22]	14.41 ± 0.59 [6 - 24]	**0.060**
Gender (M:F)	19:18	12:15	13:21	**0.547**
CAG		41.48 ± 0.49 [38 - 48]	42.79 ± 0.50 [37 - 50]	**0.069**
AOO (Years)			49.35 ± 2.05 [22 - 68]	-
PAO (Years)		53.70 ± 2.63 [32 - 75]	50.52 ± 2.47 [27 - 70]	**0.383**
CAP		335.99 ± 20.14 [165.54 - 487.56]	474.78 ± 13.41 [220.44 - 678.70]	**0.000**

Summary of subjects in this study. AOO = Age of Onset; PAO = Parental Age of Onset; CAP = CAG Age Product

ANOVA posthoc Tukey: ^ p<0.01 PMvsHD

Quantification of Htt by ELISA. Next, we used an ELISA method to quantify levels of Htt protein in the fluid fraction (supernatant) of saliva. The antibodies used recognize a region common to both the wildtype and the mutant Htt protein (Fig. 4), thus our measurements are of total Htt protein levels ("tHtt": normal plus mutant Htt protein). First, we assessed whether the saliva matrix interfered with the ELISA assay by spiking-in a known amount of recombinant Htt protein to saliva samples from two normal individuals. We found that the recovery of Htt in the saliva matrix was 91.2% +/- 4.05% and that the levels of recombinant Htt diluted linearly in saliva ($R^2 = 0.976$) (Fig. S2). Next, we analyzed whether salivary levels of tHtt protein varied over the course of the day (7 am to 11 pm), or over different days in normal individuals, to assess the reliability of measurements taken on different days or at different times from the same individuals. We compared salivary tHtt levels to salivary cortisol, which exhibits a known diurnal variation with peak levels occurring upon waking (Price, 1983). Salivary tHtt in the same normal subjects did not vary over the course of the day (Fig. 3A). In a subset of n = 40 subjects, salivary tHtt did not correlate with time of day of collection (from 10 am to 4 pm) (Fig. 3B). Furthermore, there was remarkable consistency in salivary tHtt levels measured in samples, from the same patient, over four different (Fig. 3C).

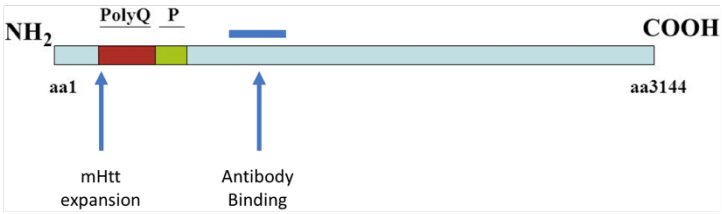

Figure 4: Schematic representation of antibody binding site on the whole Htt protein. Binding site is downstream of the polyglutamine mutant expansion, thus binding region is common to normal and mutant Htt and measures total Htt protein (tHtt). Antibodies used correspond to amino acids 802-940 of the human Htt protein.

Table 2: Clinical characteristics by diagnostic group Mean ± SEM [Range]				
	NC N = 37	**PM** N = 27	**HD** N = 34	**P-Value** ANCOVA
UHDRS TFC (0 - 13)	12.92 ± 0.05 [12 - 13]	12.81 ± 0.12 [10 - 13]	9.41 ± 0.43†^ [2 - 13]	**0.000**
UHDRS TMS (0 - 124)	1.74 ± 0.35 [0 - 5]	2.93 ± 0.53 [0 - 11]	30.18 ± 2.34†^ [10 - 63]	**0.000**
UHDRS TCS (0 - 28)	0	0.11 ± 0.08 [0 - 2]	6.26 ± 0.60†^ [1 - 17]	**0.000**
TUG (sec)	9.67 ± 0.34 [8 - 14]	9.23 ± 0.31 [7 - 12]	11.97 ± 0.68†^ [8 - 23]	**0.001**
MMSE (0 - 30)	28.96 ± 0.24 [25 - 30]	28.26 ± 0.32 [25 - 30]	26.52 ± 0.48†^ [19 - 30]	**0.000**
MoCA (0 - 30)	27.48 ± 0.27 [25 - 30]	27.22 ± 0.43 [24 - 30]	24.21 ± 0.62†^ [16 - 30]	**0.000**
SDM (0 - 110)	47.24 ± 1.84 [31 - 66]	48.48 ± 2.12 [24 - 67]	29.94 ± 1.90†^ [11 - 54]	**0.000**
PBA (0 - 160)	2.28 ± 0.68 [0 - 13]	6.67 ± 1.81 [0 - 33]	12.29 ± 2.83† [0 - 75]	**0.007**
HADS-SIS (0 - 66)	13.28 ± 1.45 [0 - 33]	20.81 ± 2.53 [2 - 53]	20.07 ± 3.06 [0 - 64]	**0.184**
Salivary tHtt (ng/mL)	0.35 ± 0.04 [0.03 - 0.78]	0.42 ± 0.05 [0.05 - 1.24]	0.51 ± 0.04† [0.20 - 1.32]	**0.022**

UHDRS = Unified Huntington's Disease Rating Scale; TFC = Total Functional Capacity; TMS = Total Motor Score; TCS = Total Chorea Score; TUG = Timed Up and Go; MMSE = Mini Mental State Exam; MoCA = Montreal Cognitive Assessment; SDM = Symbol Digit Modalities; PBA = Problem Behaviors Assessment; HADS-SIS = Hospital Anxiety and Depression Scale-Snaith Irritability Scale; tHtt = Total Huntingtin Protein Concentration

Age controlled ANCOVA posthoc Tukey:* p<0.01 NCvsPM; †p<0.01 NCvsHD; ^ p<0.01 PMvsHD

Clinical characteristics of participant groups. Our study participants were well characterized by a battery of clinical functional, motoric, cognitive, and behavioral assessments. As expected, the manifest HD group was significantly different from the premanifest HD and the

normal control groups (Table 2). Using the Unified Huntington's Disease Rating Scale (UHDRS) Total Functional Capacity (TFC) assessment, HD subjects (9.41 ± 0.43) were rated to have significantly lower functional capacity compared to PM (12.81 ± 0.12; p=0.000) and NC (12.92 ± 0.05; p=0.000) (Table 2). The UHDRS Total Motor Score (TMS) determined HD subjects (30.18 ± 2.34) had significantly greater motor symptoms compared to PM (2.93 ± 0.53; p=0.000) and NC (1.74 ± 0.35; p=0.000) (Table 2). This was also reflected in the UHDRS Total Chorea Score (TCS), a sub-score of the TMS only measuring chorea, which showed that HD subjects (6.26 ± 0.60) had significantly greater chorea compared to PM (0.11 ± 0.08; p=0.000) and NC (0.00; p=0.000). In all three cognitive assessments, the Mini Mental State Exam (MMSE), the Montreal Cognitive Assessment (MoCA), and the Symbol Digit Modalities (SDM) test, the HD subjects scored significantly lower compared to PM and NC (Table 2) indicating poorer cognition consistent with HD. Behaviorally, HD subjects scored higher on the Problem Behaviors Assessment (PBA) (12.29 ± 2.83) compared to both PM and NC, but this difference was only significant in HD (2.28 ± 0.68; p<0.01) vs NC. In all the assessments, PM individuals were indistinguishable from NC as their scores were not different at a statistically significant level (Table 2).

Salivary tHtt levels distinguish diagnostic groups. We next determined whether salivary tHtt levels were different in subjects from different diagnostic groups. Subjects were well-characterized demographically and clinically (Tables 1 and 2 respectively). Premanifest (PM) individuals were significantly younger (44.41 ± 2.41years) compared to manifest (HD) individuals (55.06 ± 2.07 years; p=0.012). We found that HD subjects had higher levels of salivary tHtt protein (0.51 ± 0.04 ng/mL) compared to NC (0.35 ± 0.04 ng/mL; p=0.022) (Fig.

5). PM subjects also had elevated levels of salivary tHtt compared to NC (0.42 ± 0.05 ng/mL)

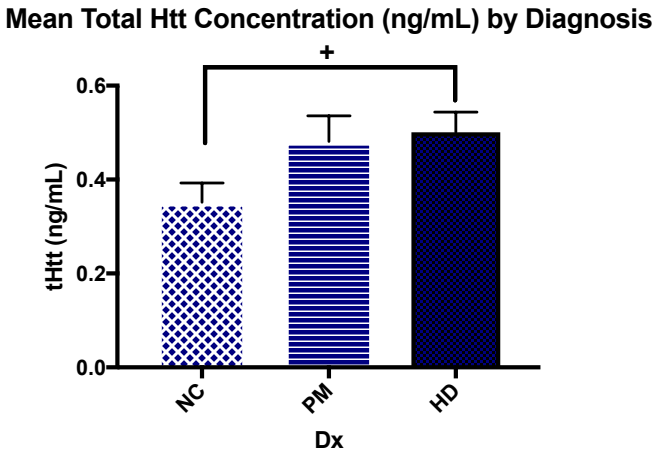

Figure 5: Mean salivary total Huntingtin (tHtt) protein concentration(ng/mL) per diagnosis group with standard error bars. +p<0.05

Dx = diagnosis; PM = premanifest HD; HD = manifest HD

(Fig. 5), but this difference did not reach statistical significance (p>0.05). We did not detect a significant effect of gender on salivary tHtt levels (Fig. 6). We did observe a moderate positive

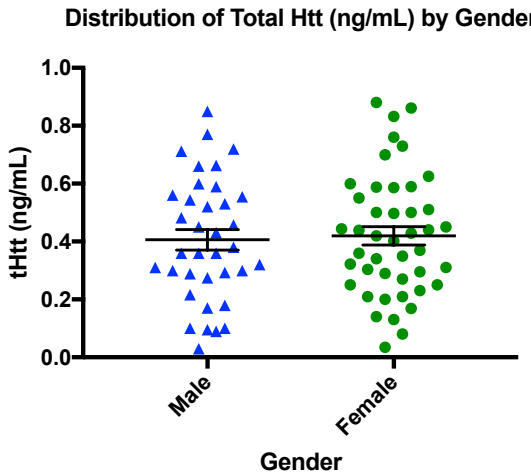

Figure 6: Distribution of salivary total Htt protein concentration(ng/mL) by gender with standard error bars

correlation between salivary tHtt level and age (r=0.418; p=0.019) in gene positive individuals with CAP>425 (Fig. 7). This correlation was also observed in HD (r=0.379; p=0.039) (Fig. S4) and all gene positive (r=0.372; p=0.006) subjects.

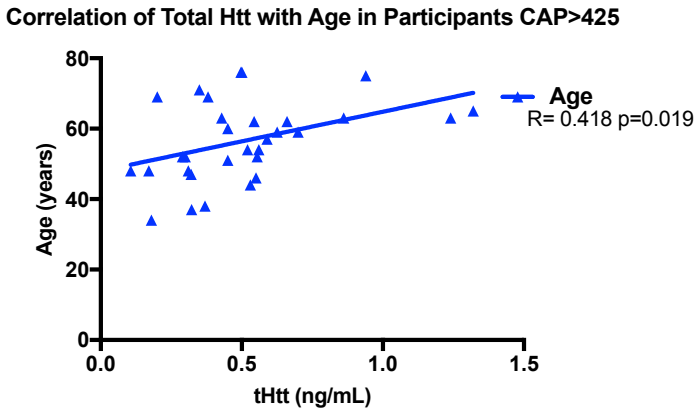

Figure 7: Correlation of salivary total Huntingtin(tHtt) protein concentration(ng/mL) with participant's age(years) in all gene positive individuals with CAP score>425

Correlations with clinical measures. We next sought to determine whether our clinical measures (including the UHDRS TMS, TCS and TFC, and the SDM test) were correlated with salivary tHtt levels in NC, PM, HD, All Gene Positive, and Gene Positive with CAP score>425 (CAP>425; n=37) subjects. Salivary tHtt showed a significant positive correlation with the UHDRS TMS score (r=0.374; p=0.038) and TCS score (r=0.361; p=0.046) (Fig. 8 A,B) in the CAP>425 group. Similar findings were observed in the All Gene Positive (Fig. S7) and HD (Fig. S8) groups, with both TMS and TCS trending toward a positive correlation. No significant correlations were seen with TMS or TCS scores in the PM (Fig. S9) or NC (Fig. S10) subjects. There was a moderate negative correlation between salivary tHtt levels and SDM scores observed in the CAP>425 (r=-0.424; p=0.017) (Fig. 8D), All Gene Positive (r=-0.293; p=0.031) (Fig. S11), and HD (r=-0.434; p=0.016) (Fig. S12) groups.

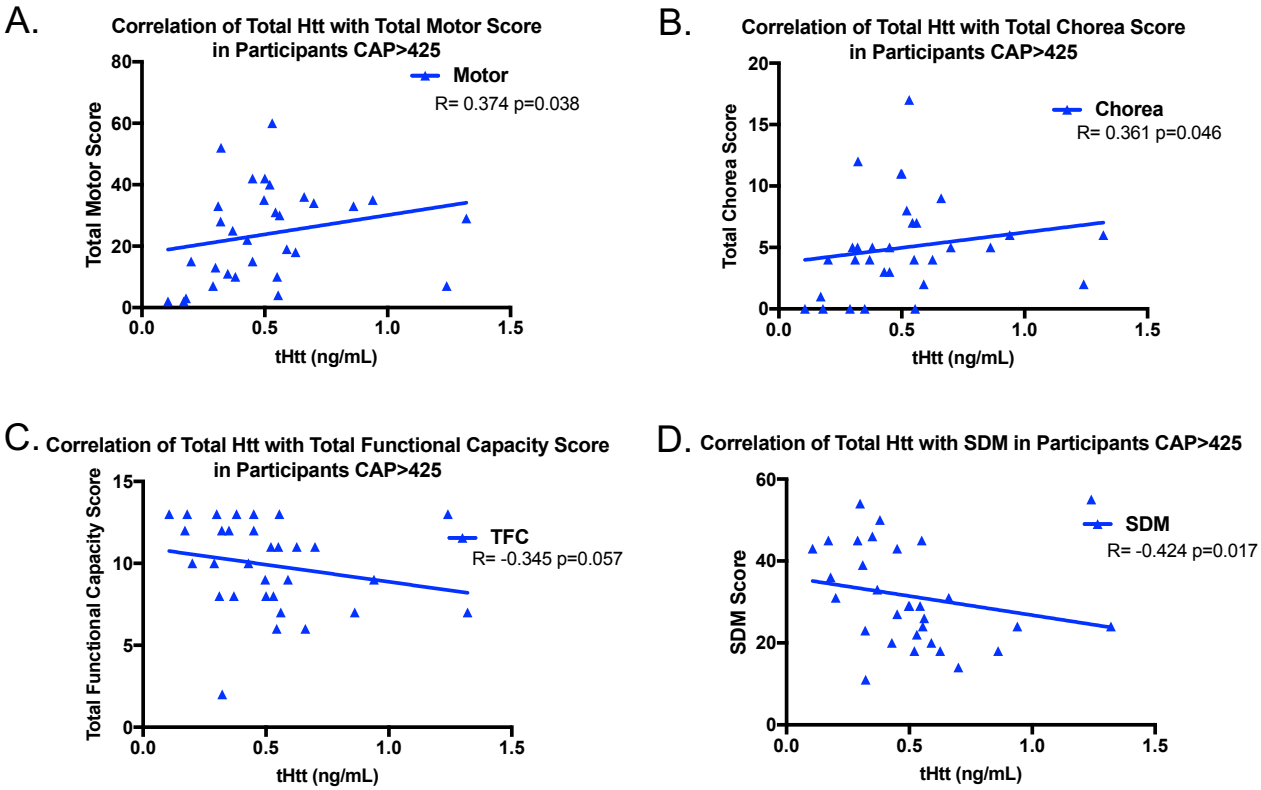

Figure 8: Spearman's r correlation of salivary total Huntingtin(tHtt) concentration(ng/mL) with several clinical measures in gene positive patients with CAP score>425(n=37). Correlation with clinical measurements of motor symptoms using the Unified Huntington's Disease Rating Scale(UHDRS): (A) total motor score (max=124) and (B) Correlation with UHDRS total chorea score (max=28). (C) Correlation with UHDRS total functional capacity score (max=13). (D) Correlation with clinical cognitive assessment symbol digit modalities(SDM) test (max=110).

Correlations between salivary and plasma tHtt. In a subset of participants (HD n=13; PM n=24; NC n=13), we were able to obtain plasma samples at the same time and date as the saliva collection. Correlations between these matched samples are seen in Figure 9. There were strong negative correlations between salivary and plasma tHtt concentration in the All Gene Positive (n=37, r=-0.632, p=0.000) (Fig. 9A); CAP>425 (n=13, r=-0.791, p=0.000) (Fig. 9B); HD (n=13, r=-0.742, p=0.004) (Fig. S18A); and PM (n=24, r=-0.661, p=0.000) (Fig. S18B) groups.

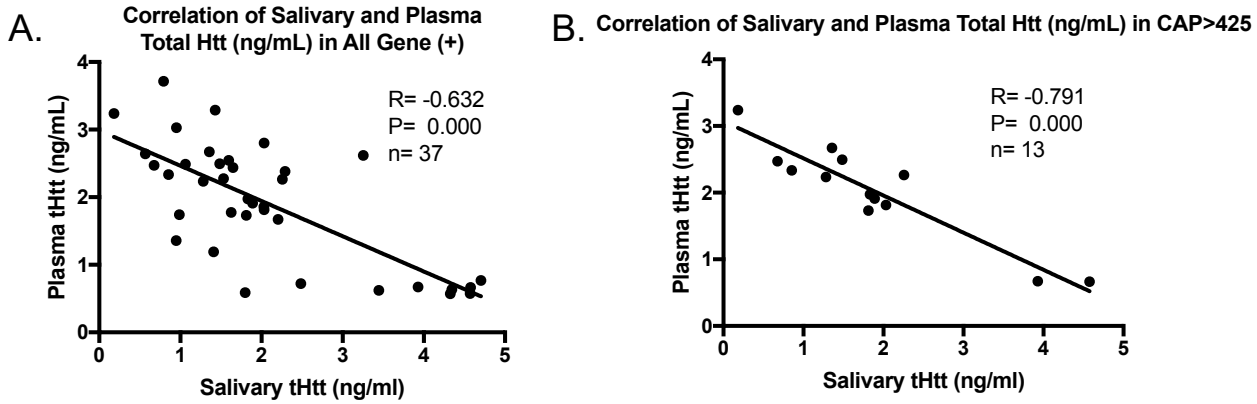

Figure 9: Correlation of salivary total Huntingtin(tHtt) protein concentration(ng/mL) and matched plasma tHtt protein concentration(ng/mL) in All Gene Positive (PM+HD; n=37) group shown in panel (A), and all gene positive with CAG age product(CAP) score >425 (n=13) group shown in panel (B). Each point represents one participant, ordered pair coordinates reflect salivary tHtt and plasma tHtt concentrations as (x,y) respectively. Saliva and plasma samples were collected from each participant at the same time, on the same day, and run on the same ELISA plate. Nonparametric correlations calculated using Spearman's r.

DISCUSSION

In HD and other neurodegenerative diseases, there is an immense need to identify biomarkers that can predict symptom onset, assess symptom severity, track disease progression, and evaluate the efficacy of potential genetic therapies. The Htt protein, which causes HD when mutated, is an ideal candidate if it can be reliably measured in peripheral tissues. In this study, we showed that tHtt protein can be detected in saliva by Western blot and that salivary tHtt can be reproducibly measured using an ELISA assay. Importantly, we found that salivary tHtt was significantly elevated in manifest HD subjects compared to controls and that salivary tHtt levels correlated with clinical measures of motor function and cognition. In addition, we found that salivary tHtt levels negatively correlated with levels of tHtt in plasma.

In recent years, saliva has been more thoroughly explored as a biospecimen for biomarker research. Proteomic studies have identified over 2,000 proteins in saliva which have been used to study immunologic, metabolic, and/or neurologic status of the human body (Pfaffe, 2011). Many salivary proteins have also been suggested as candidate markers for several diseases such as cardiovascular disease, oral cancer, and breast cancer (Pfaffe, 2011). Most excitingly, several proteins related to other neurodegenerative diseases have been measured in saliva such as amyloid beta (Lee, 2017) and tau (Shi, 2011) in Alzheimer's disease, and alpha-synuclein (Vivacqua, 2016) and DJ-1 (Devic, 2011; Masters, 2015) in Parkinson's disease, which is very promising moving forward as it shows that saliva may reveal information about diseases involving the brain.

We determined that tHtt is clearly present in saliva, which is extremely promising as saliva can be collected noninvasively and does not require skilled personnel to handle it. Previously, Htt protein, had only been measured in brain tissue, cerebrospinal fluid, and human

blood (Weiss, 2009; Wild, 2015); to our knowledge, this study is the first to measure tHtt in saliva.

We also found that salivary tHtt protein concentration is elevated in individuals with HD. These findings are consistent with prior studies measuring Htt in human cerebrospinal fluid (CSF) (Wild, 2015) and blood, buffy coat, and post mortem brain tissue (Weiss, 2009), all of which showed significantly elevated mutant Htt (mHtt) protein in HD compared to controls.

While tHtt is present in saliva, the origin of the salivary tHtt detected is unclear. As discussed previously, salivary components such as electrolytes, biomolecules, and proteins can enter the saliva from the plasma. As such, salivary tHtt levels could reflect overall circulating tHtt levels in the body. If so, the elevated levels of tHtt detected in manifest HD participants could be a result of elevated tHtt in the brain due to HD neurodegeneration. There could be several scenarios that contribute to an increase in tHtt. Increased salivary tHtt protein could reflect increased expression of mutant or normal Htt protein, as previously reported in mouse models and human HD brain tissue (Liu, 2013; Aronin, 1995). Also, abnormalities in the ubiquitin-proteasome pathway and autophagy systems, which typically degrade Htt protein intracellularly (Jia, 2012), have been previously reported in HD (Mitra, 2008) and are thought to contribute to the increased stability of mHtt in HD patients (Zhou, 2016; Mitra, 2008) which could explain an increase in tHtt levels as well. Further, it is known that mHtt is cleaved by several proteases into smaller mHtt N-terminal fragments which are more stable than the full-length protein (Lunkes, 2002). Thus, mHtt fragments persist in the cytosol and could contribute to elevated tHtt levels observed in HD participants. The tHtt protein could have leaked into the bloodstream from the brain via a compromised blood-brain barrier, which has been reported previously in HD (Drouin-Ouellet, 2015).

To further investigate this possibility, we collected paired plasma samples from a subset of our study participants. All the plasma samples were assayed using the same tHtt ELISA kit and the paired saliva and plasma samples were run at the same time. Our data actually showed a strong but inverse relationship between salivary and plasma tHtt protein concentrations. Since our ELISA antibody only measures a fragment of the Htt protein, it is possible that this inverse relationship stems from differing concentrations of this particular fragment in saliva compared to plasma. As previously mentioned, smaller fragments, rather than whole proteins, are more likely to enter the saliva from the blood, by either unaided passive diffusion, active transport, and/or ultrafiltration (Pfaffe, 2011). Interestingly, a prior study (Wild 2015) examining the relationship between CSF and plasma Htt concentrations reported no significant association; however, their data showed a trend towards a positive correlation ($r=0.454$; $p=0.14$). One possible future direction would be to examine CSF tHtt using the same ELISA that we used in this study to try and clarify the relationship between Htt protein in CSF, plasma, and saliva.

Another source of Htt in the saliva could be the cells found in saliva, such as leukocytes and buccal cells. A previous small study (7 PM and 14 HD subjects) showed elevated levels of mHtt, but not tHtt, in leukocyte blood fractions from manifest HD subjects compared to controls (Weiss, 2012); however, these authors did not find any meaningful correlations between mHtt from leukocytes and disease burden or striatal volume (Weiss, 2012), which might argue against leukocytes contributing to the majority of measured salivary tHtt protein. Salivary leukocyte concentrations fluctuate based on the presence and level of inflammation in the mouth (Calouius, 1958) and HD patients are known to have significantly more tooth decay; thus more oral inflammation (Saft, 2013). An increase in leukocyte concentration and more Htt from leukocytes in the saliva, could potentially explain higher levels of tHtt in saliva not seen in plasma.

Additional sources of Htt could be the salivary glands themselves, which are also known to express Htt protein (Marques Sousa, 2013). It is also possible that the nerves innervating the salivary glands release Htt into the saliva. Future investigations are required to identify the precise source of salivary tHtt.

The fact that tHtt levels in saliva in this study were found to be significantly associated with clinical assessment scores indicates its potential relevance to pathogenic and clinical events in the brains of patient with HD. We found significant correlations between salivary tHtt and cognitive, motor and functional assessments. These results further demonstrate the potential for salivary tHtt as a clinically relevant biomarker in HD. We expect that salivary levels of the mHtt might be even more highly correlated with clinical data, and possibly even predictive of disease symptoms. Follow-up studies specifically measuring mutant forms and different cleavage products of Htt will therefore be essential.

In summary, measurement of salivary Htt offers significant promise as a relevant, non-invasive disease biomarker for HD. Saliva samples can be collected efficiently and safely by minimally trained personnel, enabling frequent collections. Significant associations between salivary tHtt levels with measures of cognitive and motor function indicate its relevance to the clinical state of the patient; offering promise for both clinical research and therapeutic applications, particularly with regard to upcoming clinical trials involving Htt-lowering strategies.

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APPENDIX

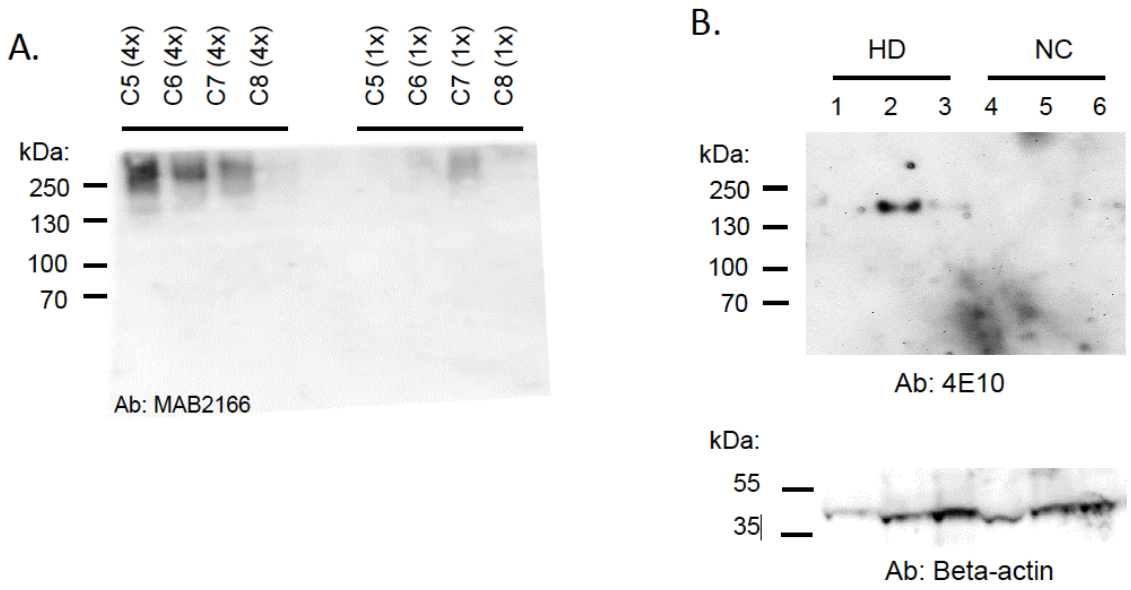


Figure S1: Western blot of Htt protein in saliva from normal and HD individuals. Saliva supernatants were used as-is (1x) or were concentrated 4-fold by vacuum centrifugation from 4 normal subjects (C5-C8) in (A), and in matched HD and control pairs (n=3) in (B). Supernatant fractions were separated by 7% SDS-PAGE and blotted onto nitrocellulose membranes for immunodetection using MAB2166 antibody (1:1,000) (A) or the 4E10 antibody (1:1,000) and beta-actin (1:1,000) (B). Gel image was acquired using a FluorochemE imager.

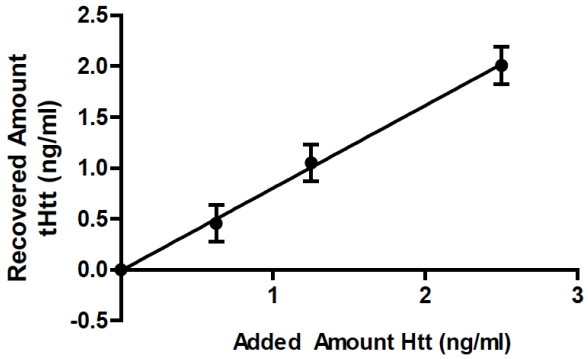


Figure S2: Linearity of dilution of tHtt in saliva. Levels of tHtt were determined by ELISA. The amount of tHtt(ng/ml) reported is relative to a recombinant Htt protein of 139 amino acids per the ELISA assay. The spiked-in recovery for the saliva matrix was 91.2% +/-4.05% and the R^2 value for linear regression = 0.976.

Table S1: Nonparametric correlations of tHtt(ng/mL) and demographic characteristics per diagnosis

	NC n=37		PM n=27		HD n=34		CAP>425 n=37	
	Spearman's r	**P-Value**	**Spearman's r**	**P-Value**	**Spearman's r**	**P-Value**	**Spearman's r**	**P-Value**
tHtt vs. Age	0.039	0.83	0.233	0.273	**0.379**	**0.039**	**0.418**	**0.019**
tHtt vs. Gender	0.051	0.780	-0.232	0.275	-0.164	0.387	-0.265	0.150
tHtt vs. Education	0.063	0.771	-0.095	0.659	-0.171	0.367	-0.287	0.117
tHtt vs. CAG			-0.356	0.087	-0.206	0.276	**-0.368**	**0.042**
tHtt vs. AOO			0.500	0.667	0.168	0.374	0.184	0.358
tHtt vs. PAO			0.143	0.505	0.200	0.297	0.075	0.688
tHtt vs. Langbehn			0.237	0.265	-0.173	0.362	-0.040	0.830
tHtt vs. Aylward			-0.227	0.286	-0.197	0.307	-0.242	0.189
tHtt vs. CAP			-0.208	0.329	0.217	0.249	0.116	0.534
tHtt vs. DBS			-0.302	0.152	0.164	0.386	-0.068	0.717

BMI = Body Mass Index; CAG = CAG repeat length; AOO = Age of Onset; PAO = Parental Age of Onset; CAP = CAG Age Product; DBS = Disease Burden Score

NC = normal control; PM = premanifest HD; HD = manifest HD; All Gene (+) = all premanifest and manifest HD participants

Table S2: Nonparametric correlations of tHtt(ng/mL) and clinical characteristics per diagnosis

	NC n=37		PM n=27		HD n=34		CAP>425 n=37	
	Spearman's r	**P-Value**	**Spearman's r**	**P-Value**	**Spearman's r**	**P-Value**	**Spearman's r**	**P-Value**
tHtt vs. UHDRS TFC	0.135	0.512	0.297	0.158	-0.306	0.100	-0.345	0.057
tHtt vs. UHDRS TMS	0.332	0.097	0.124	0.563	0.313	0.093	**0.374**	**0.038**
tHtt vs. UHDRS TCS			0.042	0.847	0.313	0.093	**0.361**	**0.046**
tHtt vs. TUG	0.133	0.566	0.135	0.529	0.384	0.058	0.286	0.157
tHtt vs. MMSE	0.067	0.744	0.042	0.845	-0.167	0.377	-0.122	0.512
tHtt vs. MoCA	-0.166	0.439	-0.233	0.273	-0.085	0.654	-0.176	0.344
tHtt vs. SDM	0.055	0.800	0.048	0.823	**-0.434**	**0.016**	**-0.424**	**0.017**
tHtt vs. PBA	-0.261	0.219	0.120	0.577	**-0.405**	**0.027**	-0.176	0.345
tHtt vs. HADS	-0.200	0.350	-0.074	0.736	-0.283	0.170	-0.324	0.093

UHDRS = Unified Huntington's Disease Rating Scale; TFC = Total Functional Capacity; TMS = Total Motor Score; TCS = Total Chorea Score; TUG = Timed Up&Go; MMSE = Mini Mental State Exam; MoCA = Montreal Cognitive Assessment; SDM = Symbol Digit Modalitites; PBA = Problem Behaviors Assessment; HADS-SIS = Hospital Anxiety and Depression Scale-Snaith Irritability Scale

NC = normal control; PM = premanifest HD; HD = manifest HD; All Gene (+) = all premanifest and manifest HD participants

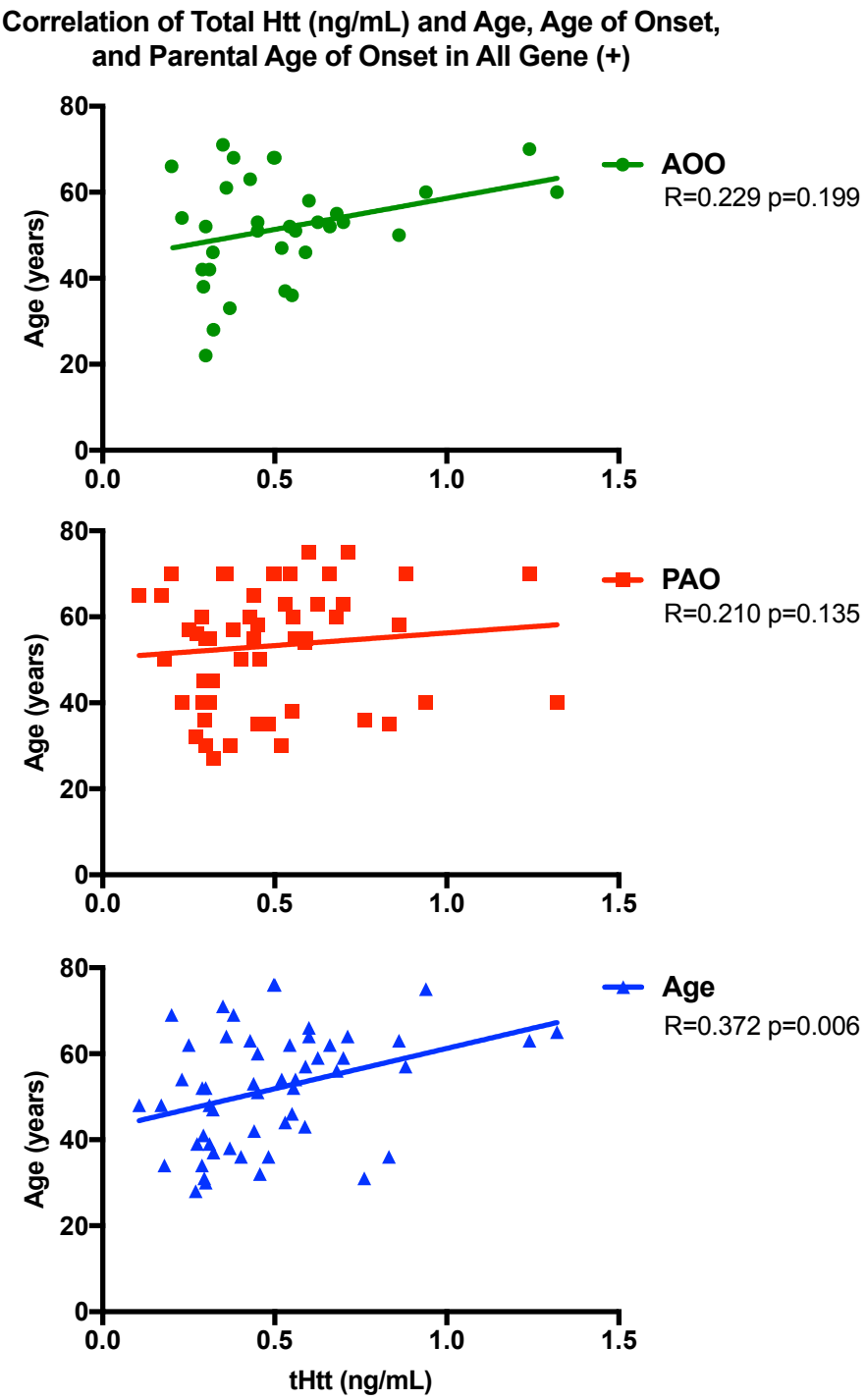


Figure S3: Correlation of total Huntingtin (tHtt) protein concentration (ng/mL) with participant's age of onset(AOO), parental age of onset(PAO), and age(years) in all gene positive participants.

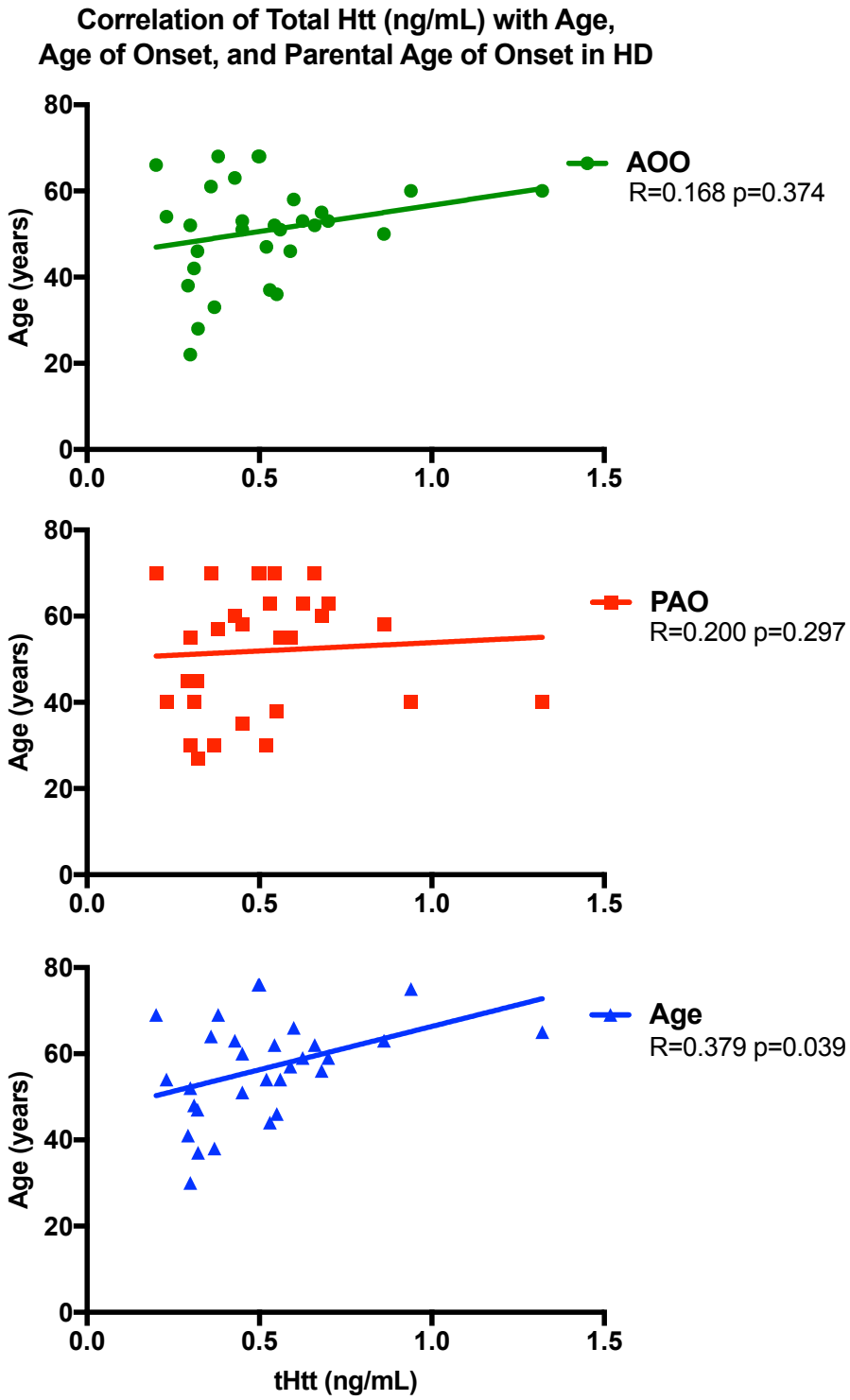


Figure S4: Correlation of total Huntingtin (tHtt) protein concentration (ng/mL) with participant's age of onset(AOO), parental age of onset(PAO), and age(years) in manifest HD participants.

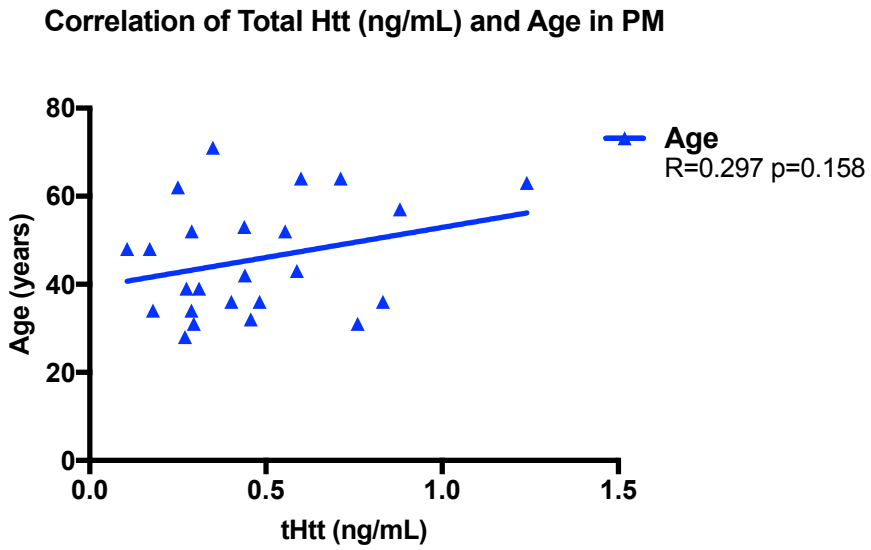


Figure S5: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's age(years) in premanifest(PM) HD participants.

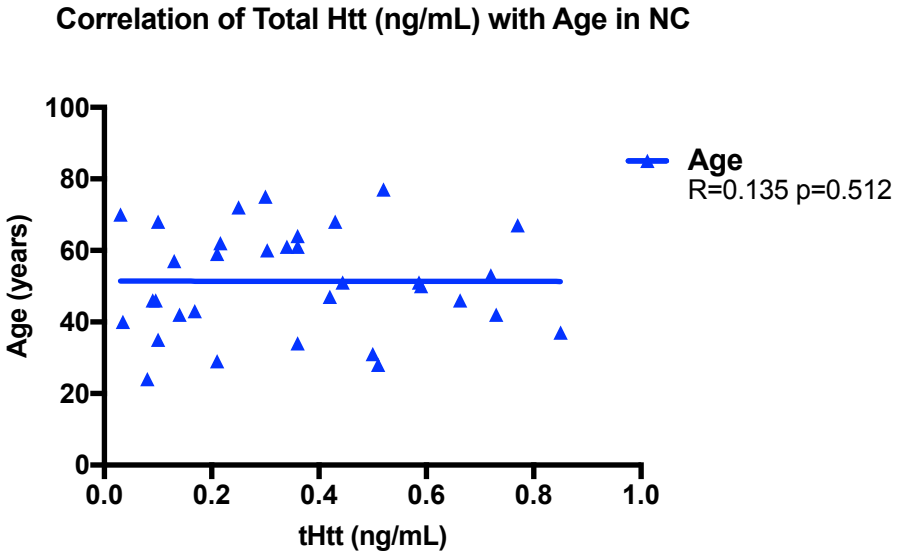


Figure S6: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's age(years) in normal control(NC) participants.

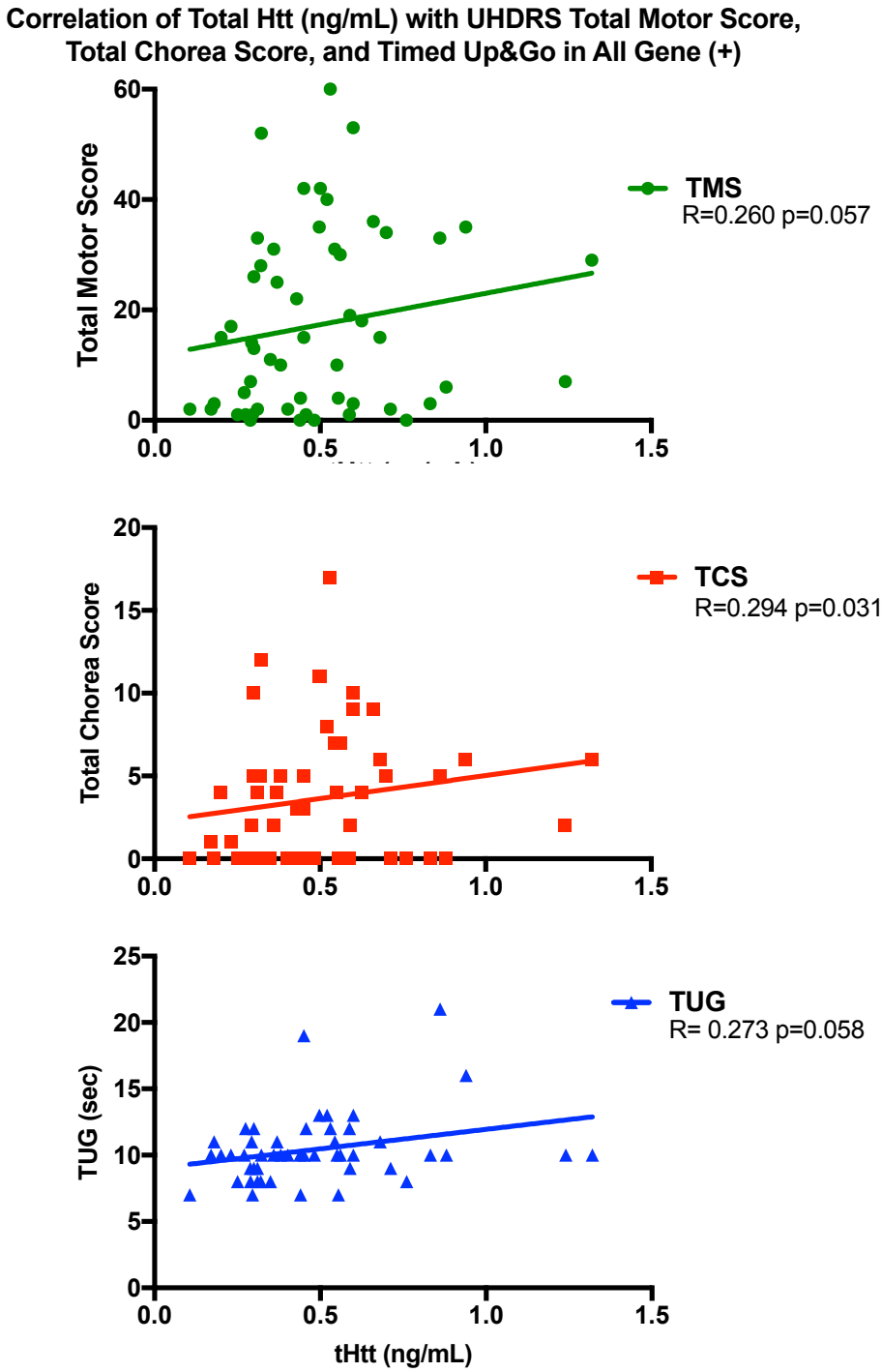


Figure S7: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's total motor score(TMS), total chorea score(TCS) on the Unified Huntington's Disease Rating Scale(UHDRS), and their timed up&go(TUG) time(sec) in all gene positive participants.

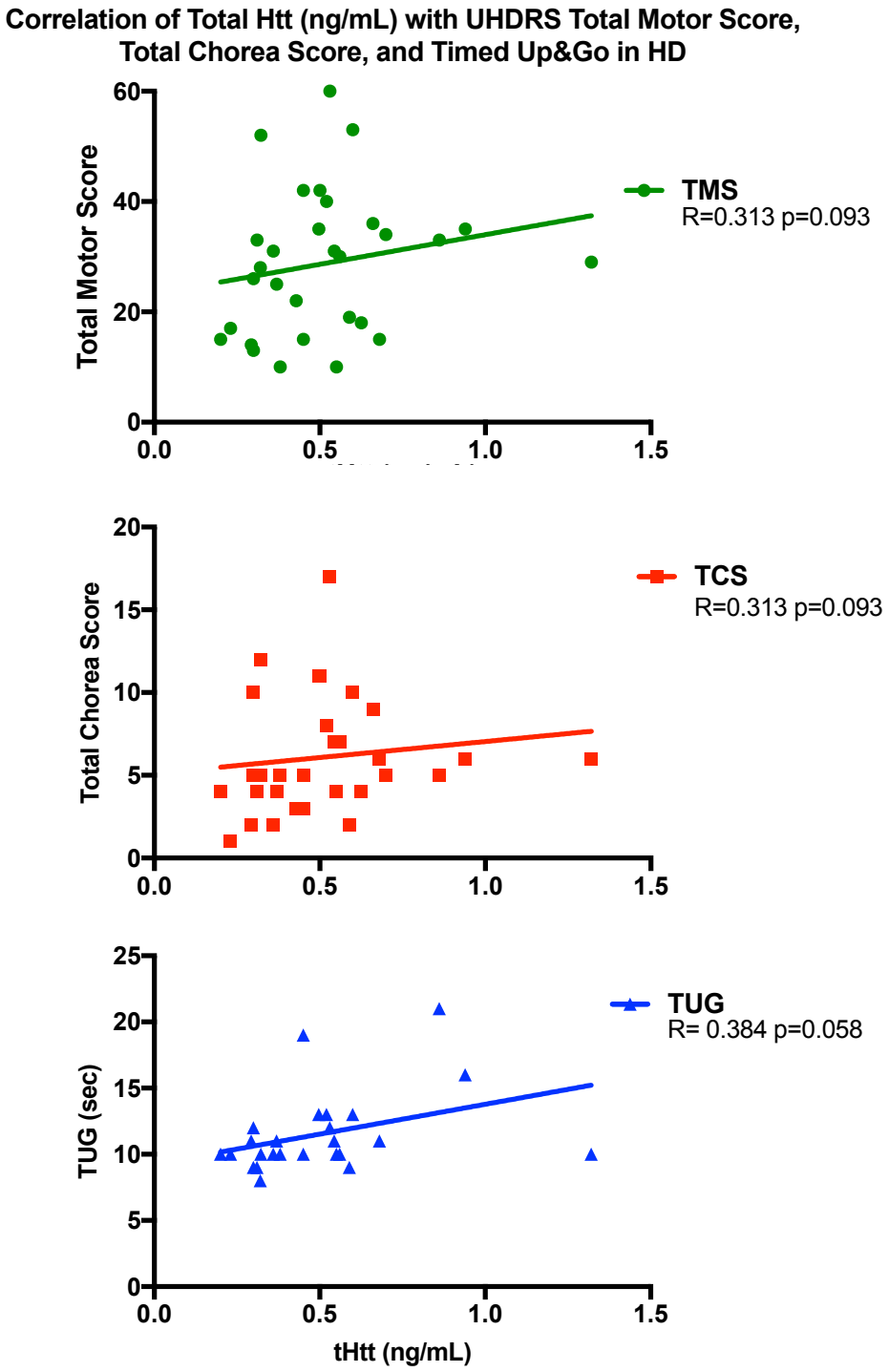


Figure S8: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's total motor score(TMS), total chorea score(TCS) on the Unified Huntington's Disease Rating Scale(UHDRS), and their timed up&go(TUG) time(sec) in manifest HD participants.

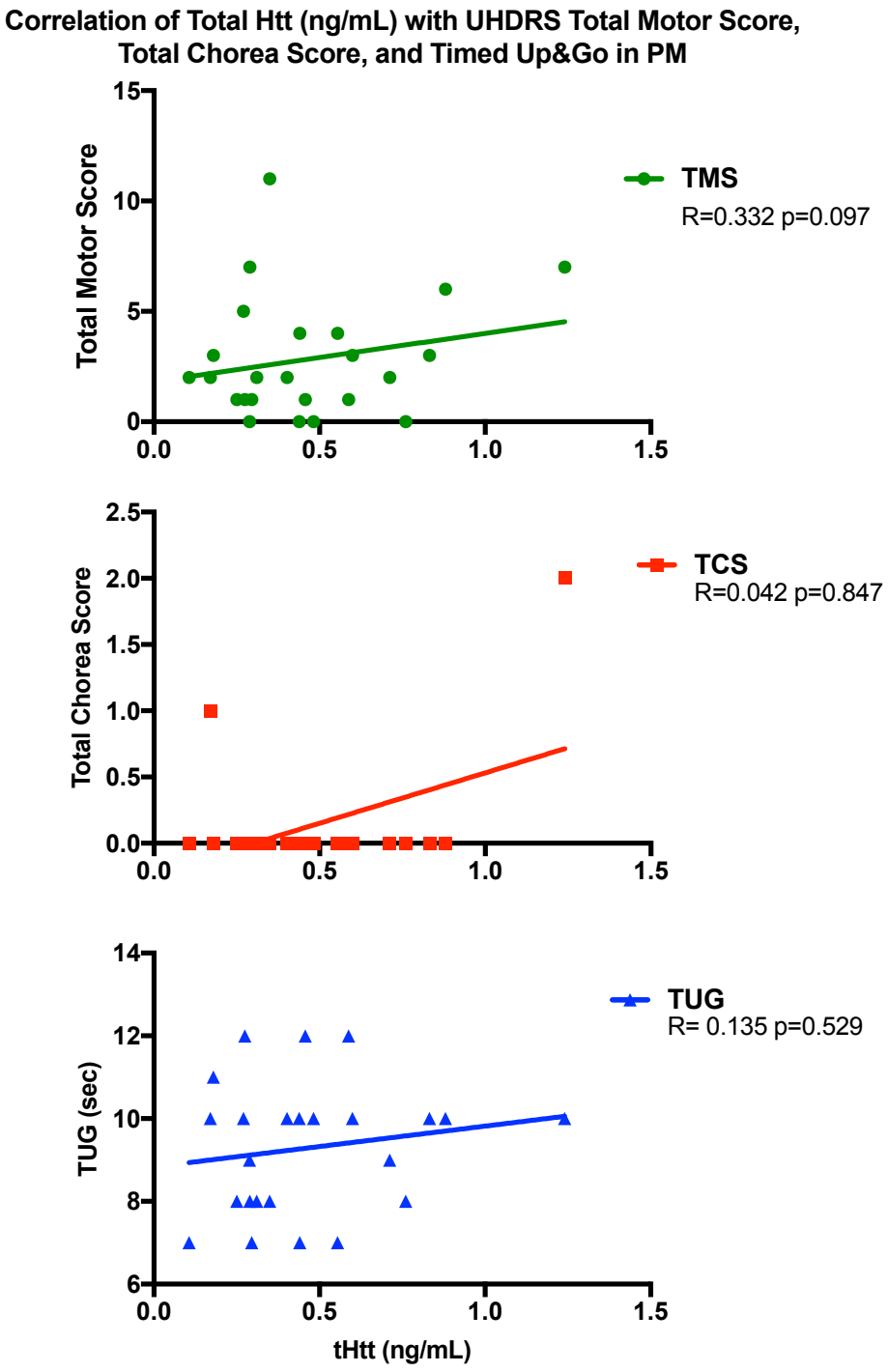


Figure S9: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's total motor score(TMS), total chorea score(TCS) on the Unified Huntington's Disease Rating Scale(UHDRS), and their timed up&go(TUG) time(sec) in premanifest(PM) HD participants.

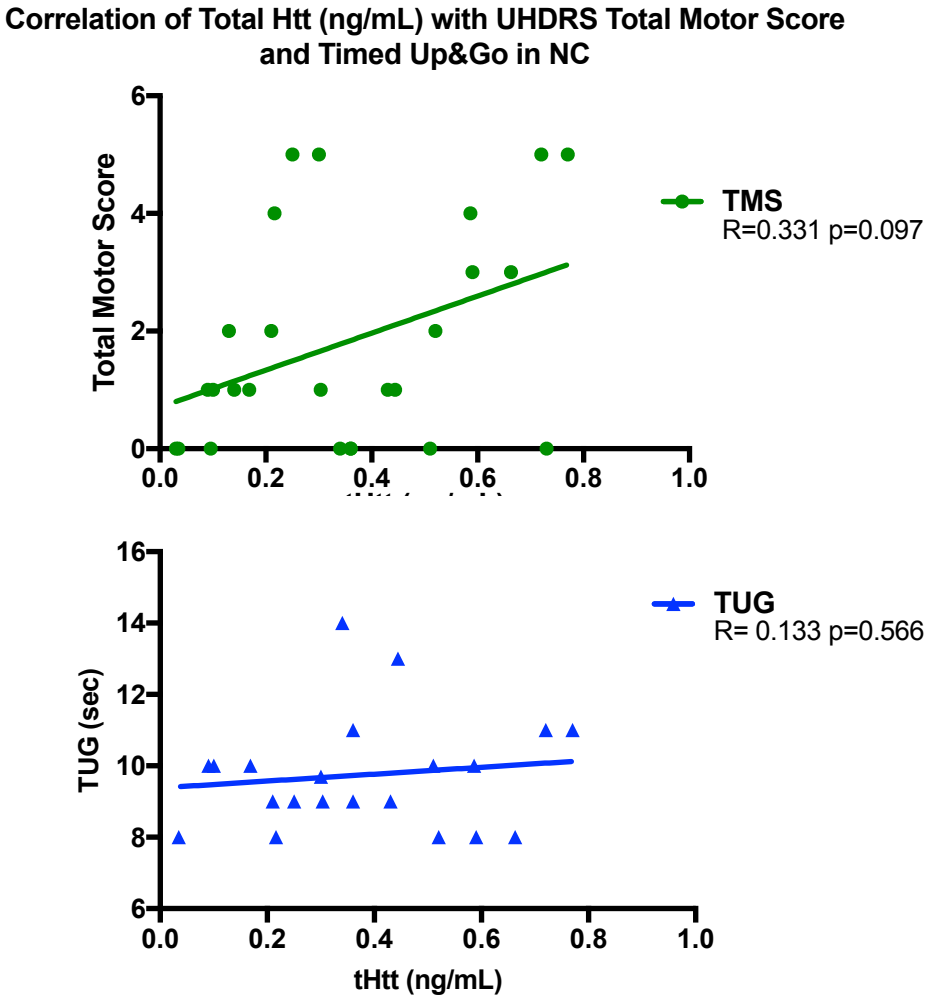


Figure S10: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's total motor score(TMS) on the Unified Huntington's Disease Rating Scale(UHDRS), and their timed up&go(TUG) time(sec) in normal control(NC) participants.

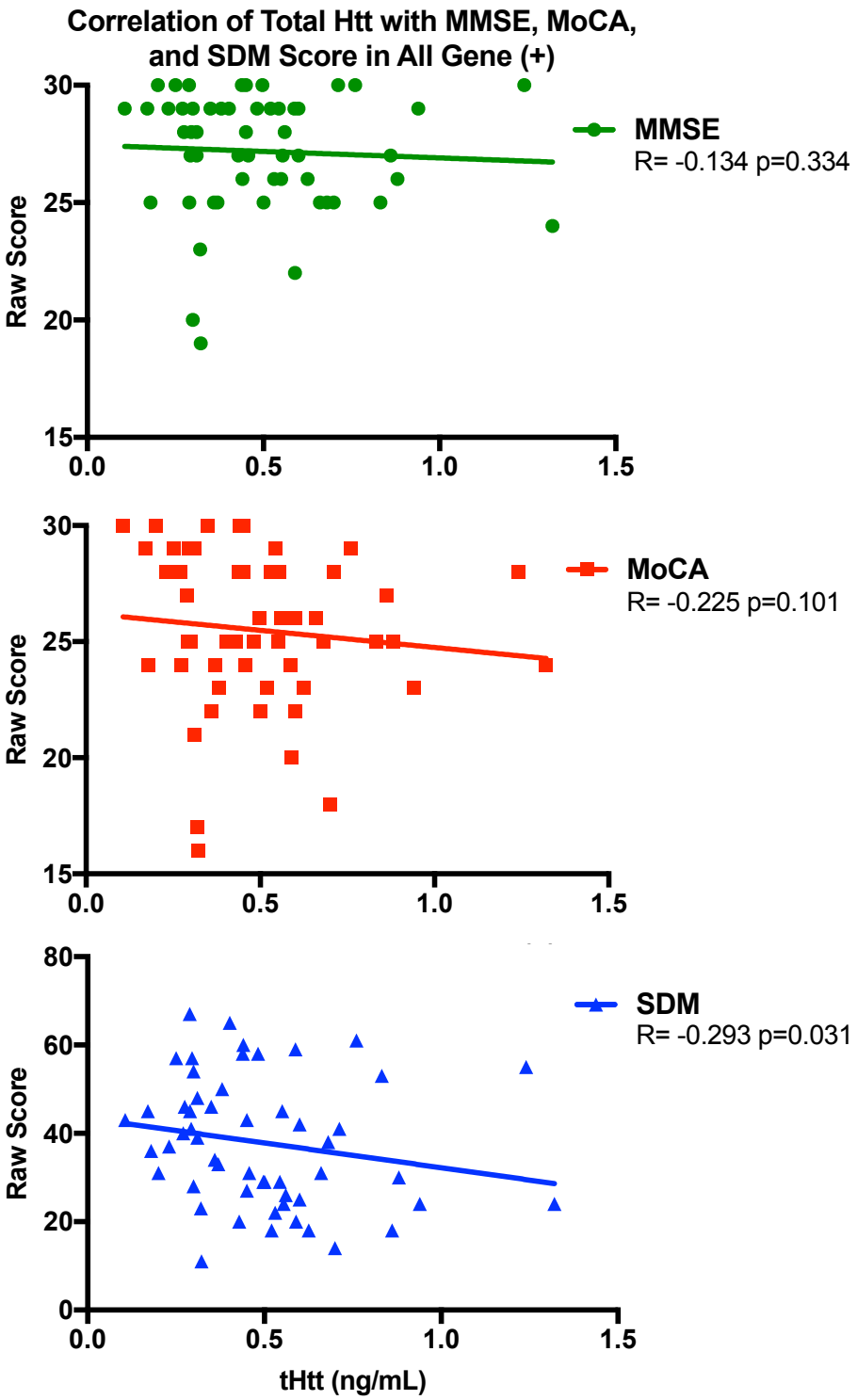


Figure S11: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Mini Mental State Exam(MMSE), Montreal Cognitive Assessment(MoCA), and Symbol Digit Modalities(SDM) scores in all gene positive participants.

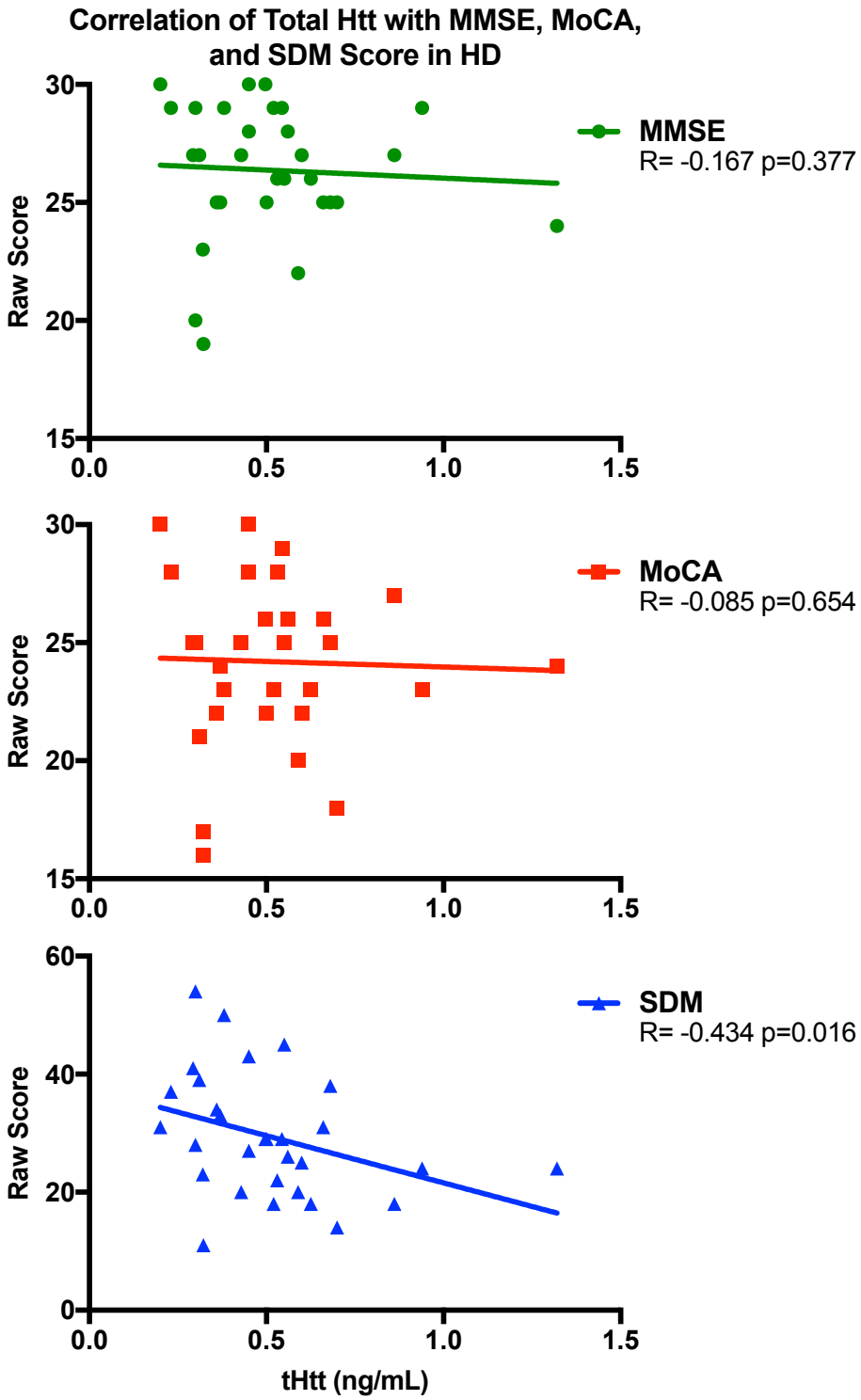


Figure S12: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Mini Mental State Exam(MMSE), Montreal Cognitive Assessment(MoCA), and Symbol Digit Modalities(SDM) scores in manifest HD participants.

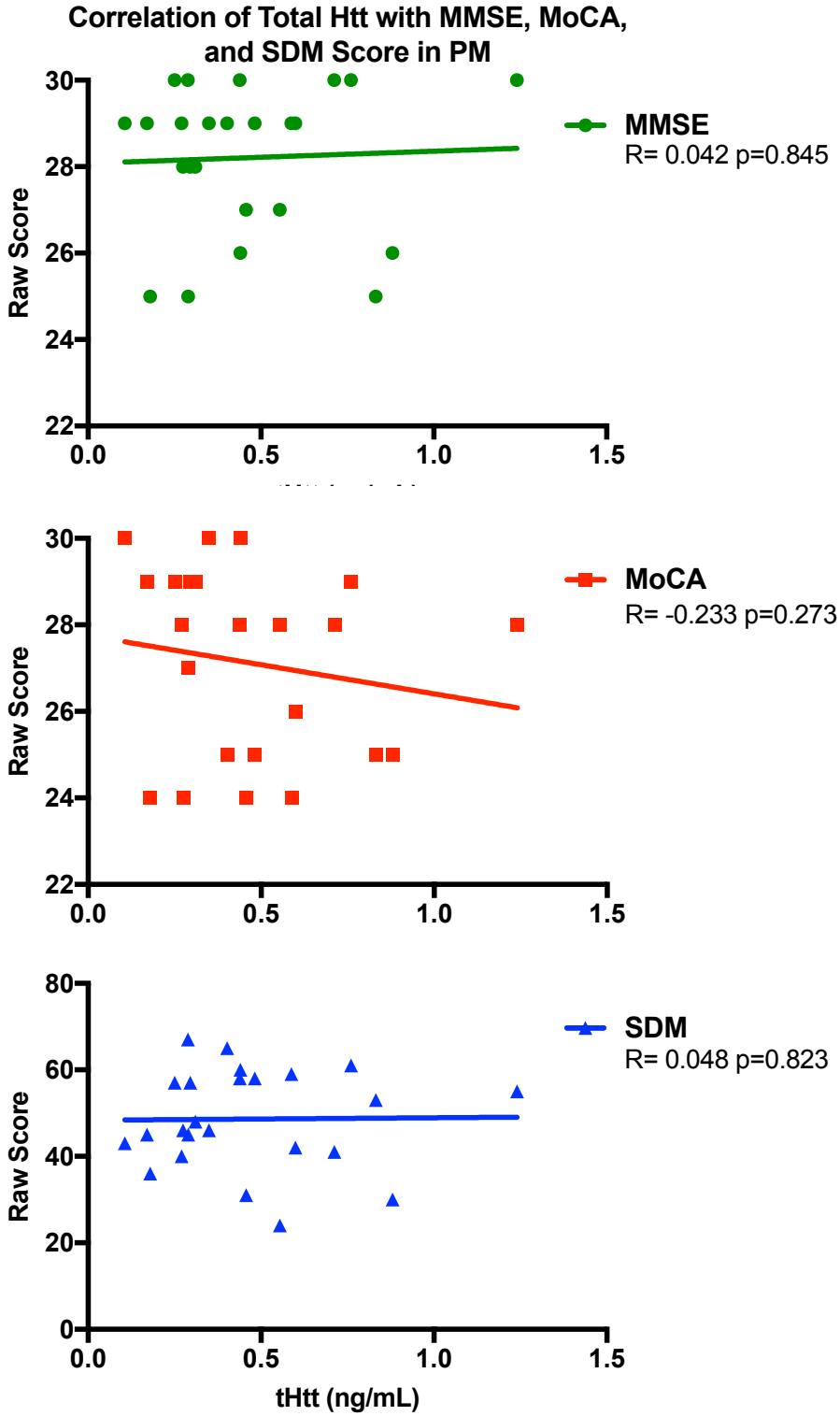


Figure S13: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Mini Mental State Exam(MMSE), Montreal Cognitive Assessment(MoCA), and Symbol Digit Modalities(SDM) scores in premanifest(PM) HD participants.

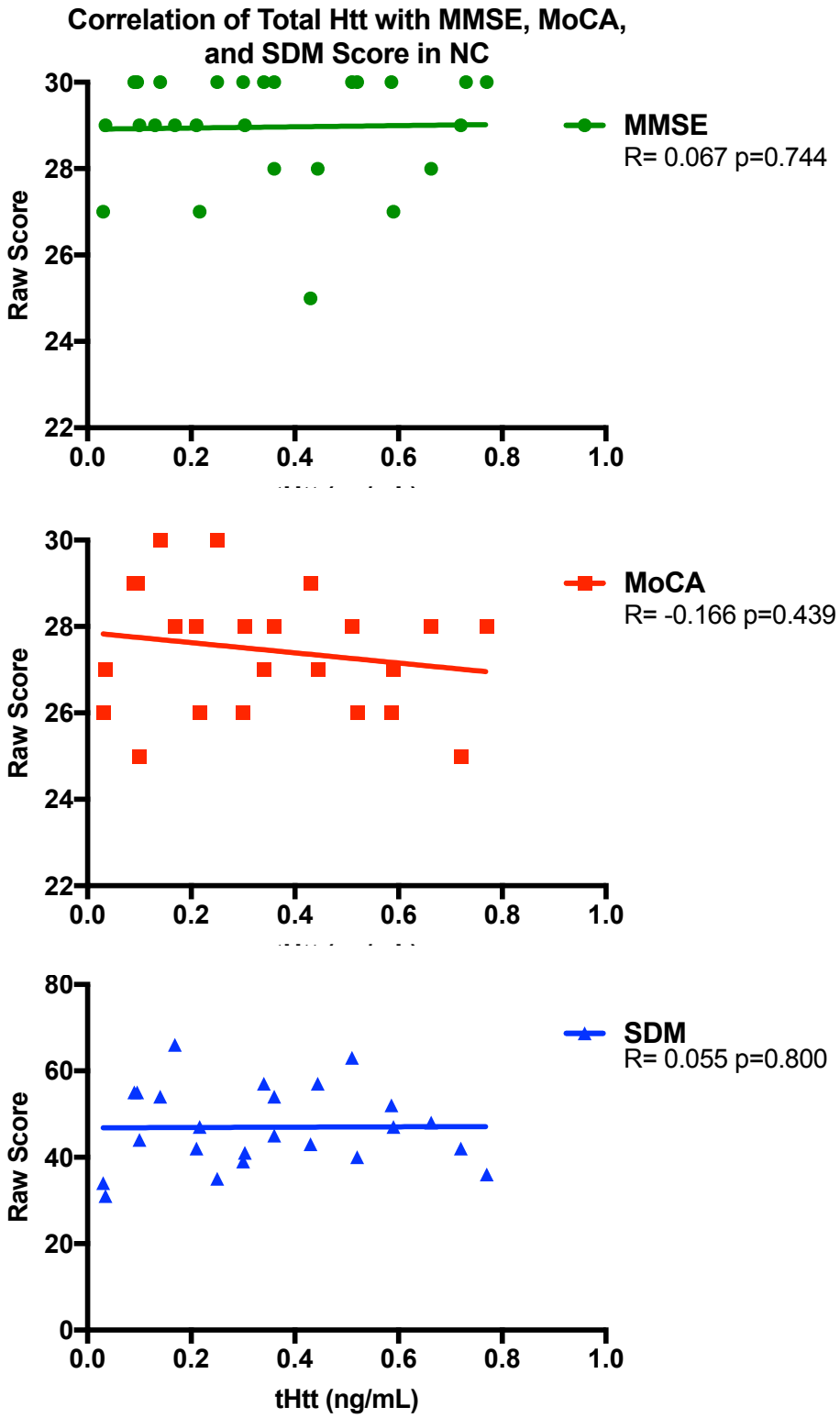


Figure S14: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Mini Mental State Exam(MMSE), Montreal Cognitive Assessment(MoCA), and Symbol Digit Modalities(SDM) scores in normal control(NC) participants.

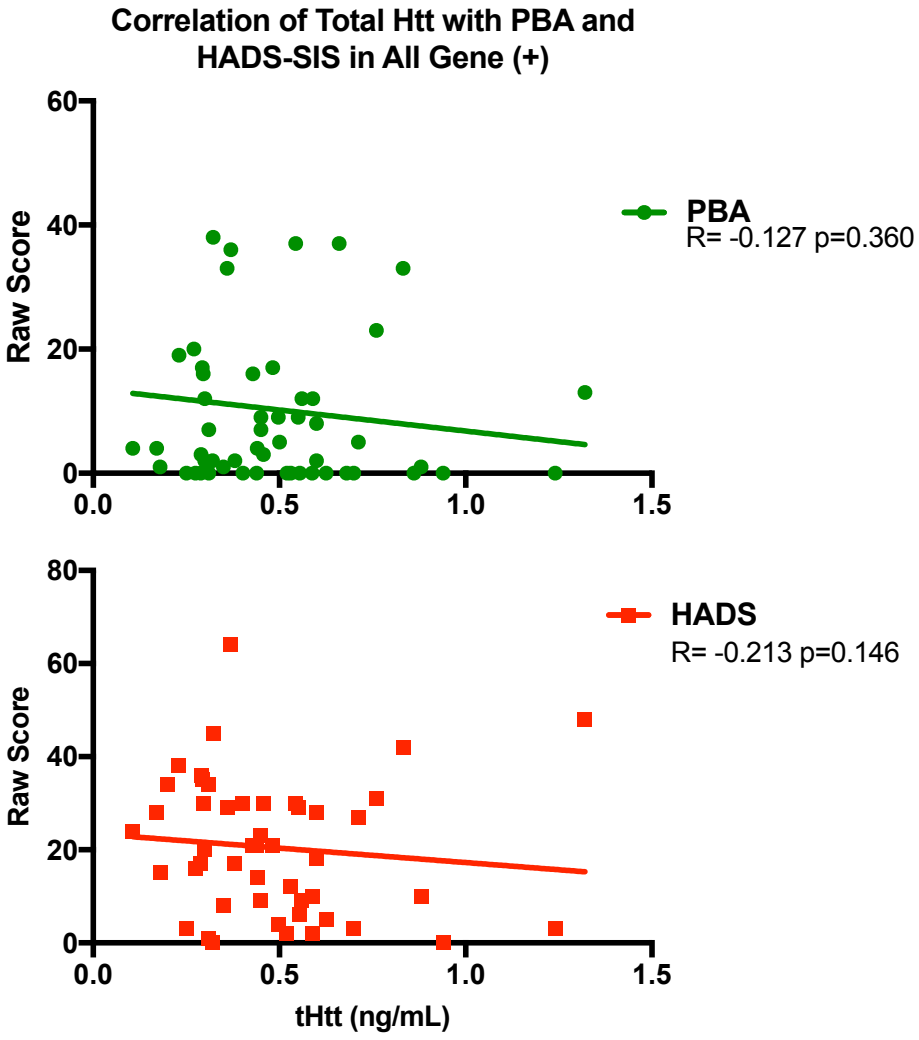


Figure S15: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Problem Behaviors Assessment(PBA) and Hospital Anxiety and Depression Scale-Snaith Irritability Scale(HADS-SIS) in all gene positive participants.

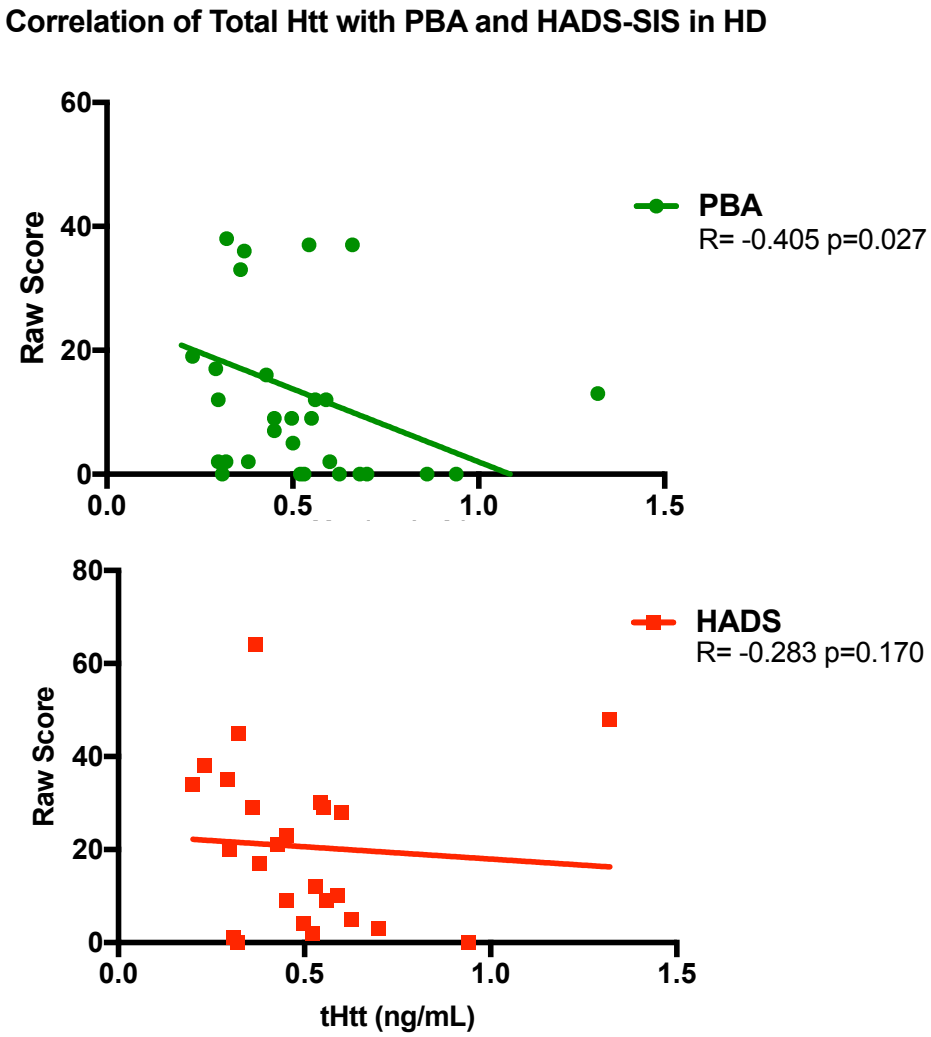


Figure S16: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Problem Behaviors Assessment(PBA) and Hospital Anxiety and Depression Scale-Snaith Irritability Scale(HADS-SIS) in manifest HD participants.

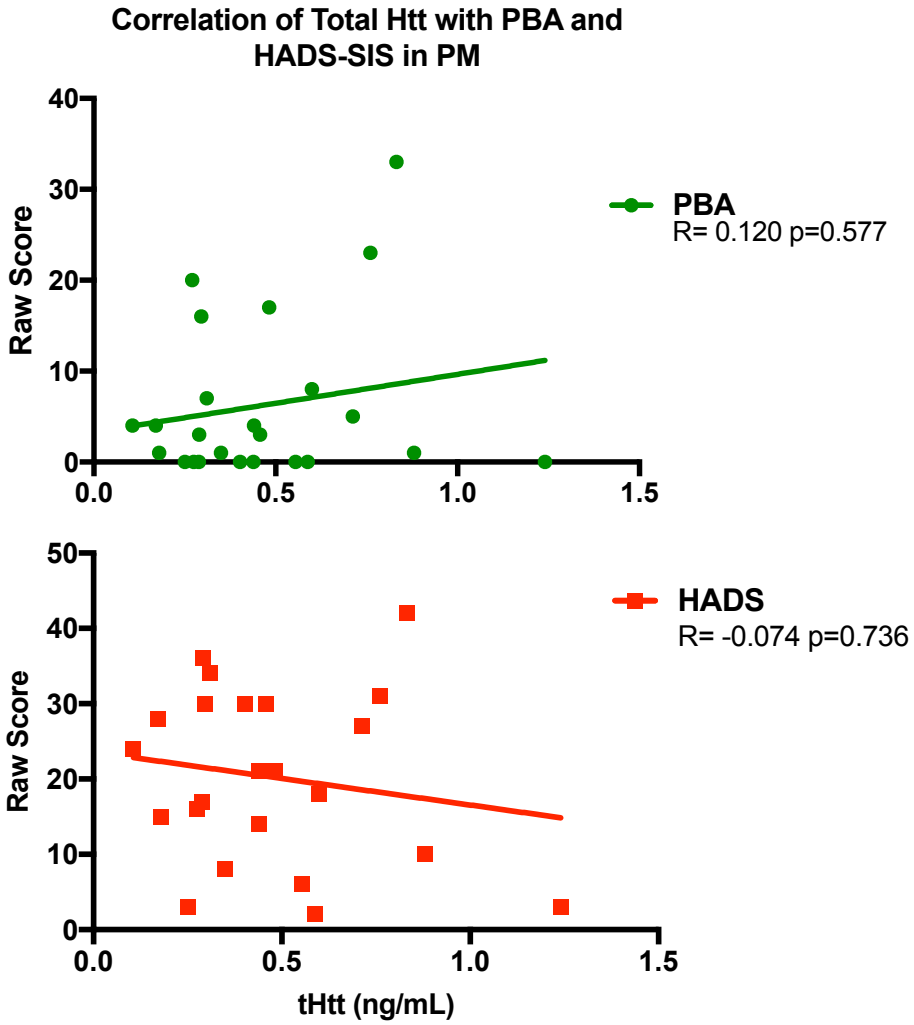


Figure S17: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Problem Behaviors Assessment(PBA) and Hospital Anxiety and Depression Scale-Snaith Irritability Scale(HADS-SIS) in premanifest(PM) HD participants.

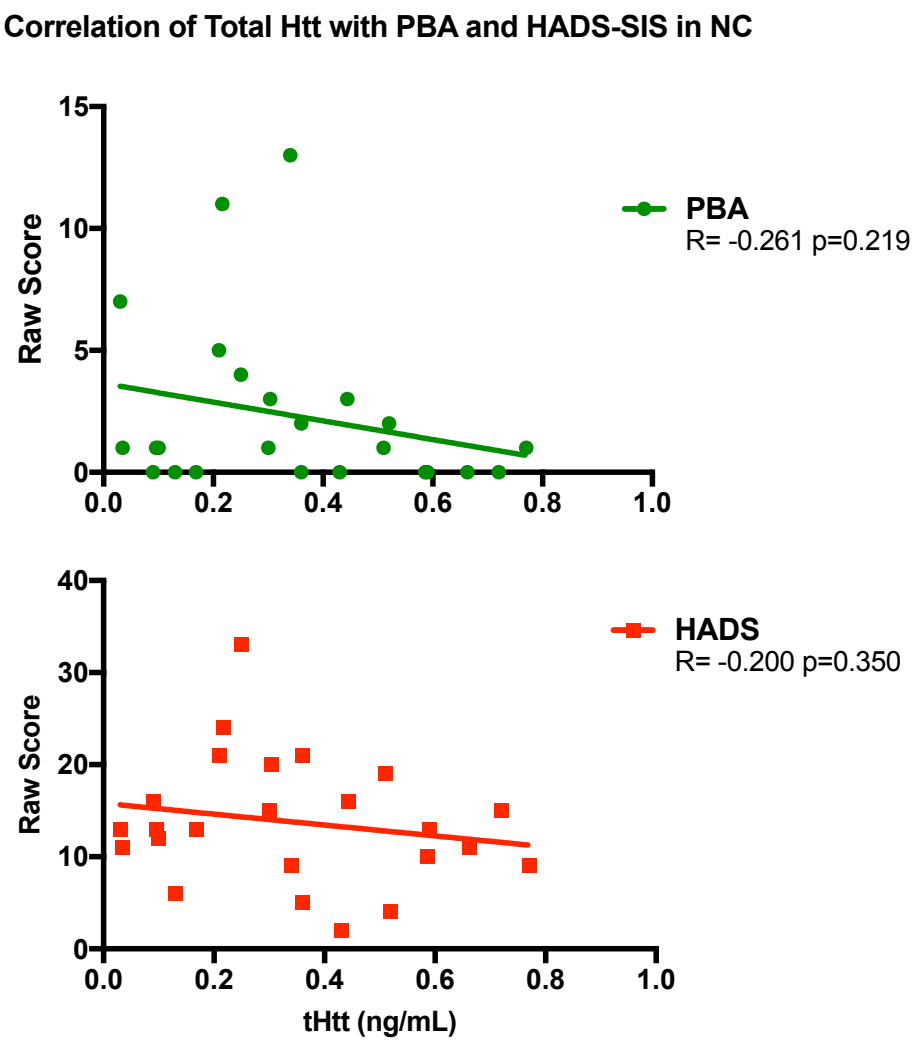
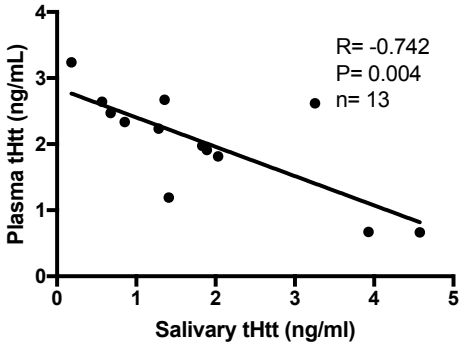
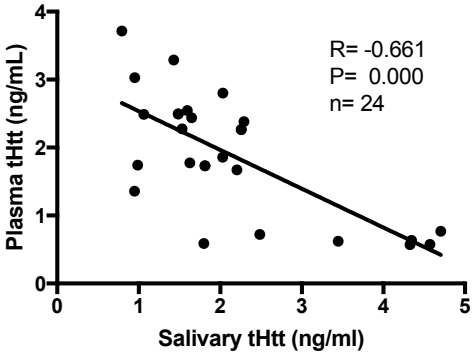


Figure S18: Correlation of total Huntingtin(tHtt) protein concentration (ng/mL) with participant's Problem Behaviors Assessment(PBA) and Hospital Anxiety and Depression Scale-Snaith Irritability Scale(HADS-SIS) in normal control(NC) participants.

A. **Correlation of Salivary and Plasma Total Htt (ng/mL) in HD**



B. **Correlation of Salivary and Plasma Total Htt (ng/mL) in PM**



C. **Correlation of Salivary and Plasma Total Htt (ng/mL) in NC**

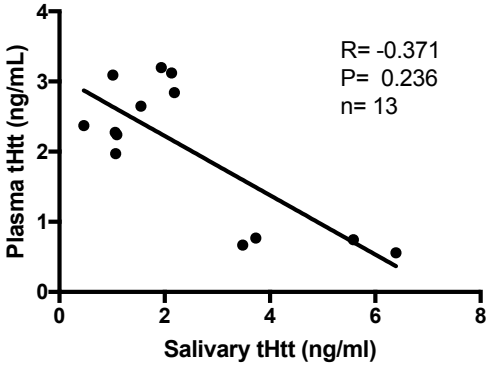


Figure S19: Correlation of participant's salivary total Huntingtin(tHtt) protein concentration (ng/mL) with his/her plasma tHtt concentration as measured by ELISA, and correlation calculated by Spearman's r in manifest HD (A), premanifest(PM) HD (B), and normal controls(NC) (C) diagnostic groups.

BIBLIOGRAPHY

1. Aronin, Neil, Kathryn Chase, Christine Young, Ellen Sapp, Cordula Schwarz, Nahida Matta, Ruth Kornreich, Bernhard Lanwehrmeyer, Edward Bird, M. Flint Beal, Jean-Paul Vonsattel, Tom Smith, Robert Carraway, Frederick M. Boyce, Anne B. Young, John B. Penney, and Marian Difiglia. "CAG Expansion Affects the Expression of Mutant Huntingtin in the Huntington's Disease Brain." Neuron. 15, 1193–201 (1995).

2. Björkqvist, Maria, Edward J. Wild, Jenny Thiele, Aurelio Silvestroni, Ralph Andre, Nayana Lahiri, Elsa Raibon, Richard V. Lee, Caroline L. Benn, Denis Soulet, Anna Magnusson, Ben Woodman, Christian Landles, Mahmoud A. Pouladi, Michael R. Hayden, Azadeh Khalili-Shirazi, Mark W. Lowdell, Patrik Brundin, Gillian P. Bates, Blair R. Leavitt, Thomas Möller, and Sarah J. Tabrizi. “A Novel Pathogenic Pathway of Immune Activation Detectable before Clinical Onset in Huntington's Disease.” The Journal of Experimental Medicine, vol. 205, no. 8, 2008, pp. 1869–1877., doi:10.1084/jem.20080178.

3. Byrne, Lauren M., Filipe B. Rodrigues, Kaj Blennow, Alexandra Durr, Blair R. Leavitt, Raymund A C Roos, Rachael I. Scahill, Sarah J. Tabrizi, Henrik Zetterberg, Douglas Langbehn, and Edward J. Wild. “Neurofilament Light Protein in Blood as a Potential Biomarker of Neurodegeneration in Huntington's Disease: A Retrospective Cohort Analysis.” The Lancet Neurology, vol. 16, no. 8, 2017, pp. 601–609., doi:10.1016/s1474-4422(17)30124-2.

4. Byrne, Lauren M., and Edward J. Wild. “Cerebrospinal Fluid Biomarkers for Huntington’s Disease.” Journal of Huntington's Disease, vol. 5, no. 1, 2016, pp. 1–13., doi:10.3233/jhd-160196.

5. Calouius, P.e.b. “The Leukocyte Count in Saliva.” Oral Surgery, Oral Medicine, Oral Pathology, vol. 11, no. 1, 1958, pp. 43–46., doi:10.1016/0030-4220(58)90219-6.

6. Corey-Bloom, Jody, Ameera Haque, Sungmee Park, Ajay Nathan, Robert Baker, and Elizabeth A. Thomas. " Salivary levels of total huntingtin are elevated in Huntington’s disease patients." Scientific Reports 2018. MS. University of California, San Diego. Web.

7. Chuang, Shih-Yi, Chih-Hung Lin, and Jia-You Fang. “Natural compounds and aging: between autophagy and inflammasome.” Biomed Res Int. 2014, 297293 (2014).

8. Devic, Ivana, Hyejin Hwang, John Scott Edgar, Kenneth Izutsu, Richard Presland, Catherine Pan, David R. Goodlett, Yu Wang, Jeff Armaly, Vitor Tumas, Cyrus P. Zabetian, James B. Leverenz, Min Shi, and Jing Zhang. “Salivary alpha-synuclein and DJ-1: potential biomarkers for Parkinson’s disease.” Brain. 134, e178 (2011).

9. Drouin-Ouellet, Janelle, Stephen J. Sawiak, Giulia Cisbani, Marie Lagacé, Wei-Li Kuan, Martine Saint-Pierre, Richard J. Dury, Wael Alata, Isabelle St-Amour, Sarah L. Mason, Frédéric Calon, Steve Lacroix, Penny A. Gowland, Susan T. Francis, Roger A. Barker, and Francesca Cicchetti. "Cerebrovascular and Blood-brain Barrier Impairments in Huntington's Disease: Potential Implications for Its Pathophysiology." Ann Neurol. 78, 160–77 (2015).

10. Bates, Gillian P., Ray Dorsey, James F. Gusella, Michael R. Hayden, Chris Kay, Blair R. Leavitt, Martha Nance, Christopher A. Ross, Rachael I. Scahill, Ronald Wetzel, Edward J. Wild, and Sarah J. Tabrizi. "Huntington Disease." Nature Reviews Disease Primers, 1:15005 EP –, 04 (2015).

11. Harrington, Deborah Lynn, Megan M. Smith, Ying Zhang, Noelle E. Carlozzi, and Jane S. Paulsen. "Cognitive Domains That Predict Time to Diagnosis in Prodromal Huntington Disease." Journal of neurology, neurosurgery, and psychiatry 83.6 (2012): 10.1136/jnnp–2011–301732. PMC.

12. Javaid, Mohammad A., Ahad S. Ahmed, Robert Durand, and Simon D. Tran. "Saliva as a Diagnostic Tool for Oral and Systemic Diseases.", Journal of Oral Biology and Craniofacial Research 6.1 (2016): 66–75. PMC.

13. Jia, Haiqun, Ryan J. Kast, Joan S. Steffan, and Elizabeth A. Thomas. "Selective Histone Deacetylase (HDAC) Inhibition Imparts Beneficial Effects in Huntington's Disease Mice: Implications for the Ubiquitin–proteasomal and Autophagy Systems." Hum Mol Genet. 21(24), 5280–5293 (2012).

14. Kwan, Wanda, Ulrike Träger, Dimitrios Davalos, Austin Chou, Jill Bouchard, Ralph Andre, Aaron Miller, Andreas Weiss, Flaviano Giorgini, Christine Cheah, Thomas Möller, Nephi Stella, Katerina Akassoglou, Sarah J. Tabrizi, and Paul J. Muchowski. "Mutant Huntingtin Impairs Immune Cell Migration in Huntington Disease." Journal of Clinical Investigation, vol. 122, no. 12, 2012, pp. 4737–4747., doi:10.1172/jci64484.

15. Lee, Jean W., Viswanath Devanarayan, Yu Chen Barrett, Russell Weiner, John Allinson, Scott Fountain, Stephen Keller, Ira Weinryb, Marie Green, Larry Duan, James A. Rogers, Robert Millham, Peter J. O'brien, Jeff Sailstad, Masood Khan, Chad Ray, and John A. Wagner. "Fit-for-Purpose Method Development and Validation for Successful Biomarker Measurement." Pharm Res. 23, 312–28 (2006).

16. Lee, Moonhee, Jian-Ping Guo, Krista Kennedy, Edith G. Mcgeer, and Patrick L. Mcgeer. "A Method for Diagnosing Alzheimer's Disease Based on Salivary Amyloid-β Protein 42 Levels." J Alzheimers Dis. 55, 1175–1182 (2017).

17. Liu, Wanzhao, Joanna Chaurette, Edith L. Pfister, Lori A. Kennington, Kathryn O. Chase, Jocelyn Bullock, Jean Paul G. Vonsattel, Richard L.M. Faull, Douglas

Macdonald, Marian DiFiglia, Phillip D. Zamore, and Neil Aronin. Increased Steady-State Mutant Huntingtin mRNA in Huntington's Disease Brain. J Huntington's Dis. 2, 491–500 (2013).

18. Lunkes, Astrid, Katrin S. Lindenberg, Léa Ben-Haïem, Chantal Weber, Didier Devys, G.bernhard Landwehrmeyer, Jean-Louis Mandel, and Yvon Trottier. "Proteases Acting on Mutant Huntingtin Generate Cleaved Products That Differentially Build Up Cytoplasmic and Nuclear Inclusions." Mol Cell. 10, 259–69 (2002).

19. Douglas, Macdonald, Beth Borowsky, Jonathan Bard, Roger Cachope, Larry Park, John Wityak, Valentina Dilda, Andrew Wood, and Ladislav Mrzljak. Pharmacodynamic biomarkers for HTT-lowering therapies: a white paper. CHDI Management/CHDI Foundation, (2015).

20. Marques Sousa and Cristovao, Sandrine Humbert. Huntingtin: here, there, everywhere! J Huntington's Dis. 2, 395–403 (2013).

21. Martinez-Horta, Saul, Jesus Perez-Perez, Erik Van Duijn, Ramon Fernandez-Bobadilla, Mar Carceller, Javier Pagonabarraga, Berta Pascual-Sedano, Antonia Campolongo, Jesus Ruiz-Idiago, Frederic Sampedro, G.bernhard Landwehrmeyer, and Jaime Kulisevsky. "Neuropsychiatric Symptoms Are Very Common in Premanifest and Early Stage Huntington's Disease." Parkinsonism & Related Disorders, vol. 25, 2016, pp. 58–64., doi:10.1016/j.parkreldis.2016.02.008.

22. Masters, Joseph M., Alastair J. Noyce, Thomas T. Warner, Gavin Giovannoni, and Gordon B. Proctor. "Elevated Salivary Protein in Parkinson's Disease and Salivary DJ-1 as A potential Marker of Disease Severity." Parkinsonism Relat Disord. 21, 1251–5 (2015).

23. Mitra, Siddhartha, and Steven Finkbeiner. "The Ubiquitin-Proteasome Pathway in Huntington's Disease." ScientificWorldJournal. 8, 421–33 (2008).

24. Ramdzan, Yasmin M., Mikhail M. Trubetskov, Angelique R. Ormsby, Estella A. Newcombe, Xiaojing Sui, Mark J. Tobin, Marie N. Bongiovanni, Sally L. Gras, Grant Dewson, Jason M.l. Miller, Steven Finkbeiner, Nagaraj S. Moily, Jonathan Niclis, Clare L. Parish, Anthony W. Purcell, Michael J. Baker, Jacqueline A. Wilce, Saboora Waris, Diana Stojanovski, Till Böcking, Ching-Seng Ang, David B. Ascher, Gavin E. Reid, and Danny M. Hatters. "Huntingtin Inclusions Trigger Cellular Quiescence, Deactivate Apoptosis, and Lead to Delayed Necrosis." Cell Reports, vol. 19, no. 5, 2017, pp. 919–927., doi:10.1016/j.celrep.2017.04.029.

25. Reiner, Anton, Ioannis Dragatsis, Scott Zeitlin, and Daniel Goldowitz. "Wild-Type Huntingtin Plays a Role in Brain Development and Neuronal Survival." Molecular Neurobiology, vol. 28, no. 3, 2003, pp. 259–276., doi:10.1385/mn:28:3:259.

26. Price, D. A., G. C. Close, and B. A. Fielding. "Age of Appearance of Circadian Rhythm in Salivary Cortisol Values in Infancy." Arch Dis Child. 58, 454–6 (1983).

27. Ross, Christopher A., Elizabeth H. Aylward, Edward J. Wild, Douglas R. Langbehn, Jeffrey D. Long, John H. Warner, Rachael I. Scahill, Blair R. Leavitt, Julie C. Stout, Jane S. Paulsen, Ralf Reilmann, Paul G. Unschuld, Alice Wexler, Russell L. Margolis, and Sarah J. Tabrizi. "Huntington Disease: Natural History, Biomarkers and Prospects for Therapeutics." Nature Reviews Neurology, vol. 10, no. 4, Nov. 2014, pp. 204–216., doi:10.1038/nrneurol.2014.24.

28. Saft, Carsten, Jürgen E. Andrich, Thomas Müller, Julia Becker, and Jochen Jackowski. "Oral and Dental Health in Huntington‘s Disease - an Observational Study." *BMC Neurology* 13 (2013): 114. *PMC*.

29. Saudou, Frederic, and Sandrine Humbert. “The Biology of Huntingtin.” Neuron, vol. 89, no. 5, 2016, pp. 910–926., doi:10.1016/j.neuron.

30. Shi, Min, Yu-Ting Sui, Elaine R. Peskind, Ge Li, Hyejin Hwang, Ivana Devic, Carmen Ginghina, John Scott Edgar, Catherine Pan, David R. Goodlett, Amy R. Furay, Luis F. Gonzalez-Cuyar, and Jing Zhang. "Salivary Tau Species Are Potential Biomarkers of Alzheimer's Disease." J Alzheimers Dis. 27, 299–305 (2011).

31. Tabrizi, Sarah J., Douglas R. Langbehn, Blair R. Leavitt, Raymund Ac Roos, Alexandra Durr, David Craufurd, Christopher Kennard, Stephen L. Hicks, Nick C. Fox, Rachael I. Scahill, Beth Borowsky, Allan J. Tobin, H. Diana Rosas, Hans Johnson, Ralf Reilmann, Bernhard Landwehrmeyer, and Julie C. Stout. "Biological and Clinical Manifestations of Huntington's Disease in the Longitudinal TRACK-HD Study: Cross-sectional Analysis of Baseline Data." The Lancet Neurology, vol. 8, no. 9, 2009, pp. 791–801., doi:10.1016/s1474-4422(09)70170-x.

32. Vivacqua, Giorgio, Anna Latorre, Antonio Suppa, Michela Nardi, Sara Pietracupa, Romina Mancinelli, Giovanni Fabbrini, Carlo Colosimo, Eugenio Gaudio, and Alfredo Berardelli. "Abnormal Salivary Total and Oligomeric Alpha-Synuclein in Parkinson’s Disease." PLoS One. 11, e0151156 (2016).

33. Weiss, Andreas, Ulrike Träger, Edward J. Wild, Stephan Grueninger, Ruth Farmer, Christian Landles, Rachael I. Scahill, Nayana Lahiri, Salman Haider, Douglas Macdonald, Chris Frost, Gillian P. Bates, Graeme Bilbe, Rainer Kuhn, Ralph Andre, and Sarah J. Tabrizi. "Mutant Huntingtin Fragmentation in Immune Cells Tracks Huntington’s Disease Progression." J Clin Invest. 122, 3731–6 (2012).

34. Weiss, Andreas, Dorothée Abramowski, Miriam Bibel, Ruth Bodner, Vanita Chopra, Marian Difiglia, Jonathan Fox, Kimberly Kegel, Corinna Klein, Stephan Grueninger, Steven Hersch, David Housman, Etienne Régulier, H. Diana Rosas, Muriel Stefani, Scott Zeitlin, Graeme Bilbe, and Paolo Paganetti. "Single-step Detection of Mutant Huntingtin

in Animal and Human Tissues: A Bioassay for Huntington's Disease." Analytical Biochemistry, vol. 395, no. 1, 2009, pp. 8–15., doi:10.1016/j.ab.2009.08.001.

35. Wild, Edward J., and Sarah J. Tabrizi. "Targets for Future Clinical Trials in Huntington's Disease: What's in the Pipeline?" Movement Disorders, vol. 29, no. 11, 2014, pp. 1434–1445., doi:10.1002/mds.26007.

36. Wild, Edward J., Roberto Boggio, Douglas Langbehn, Nicola Robertson, Salman Haider, James R.c. Miller, Henrik Zetterberg, Blair R. Leavitt, Rainer Kuhn, Sarah J. Tabrizi, Douglas Macdonald, and Andreas Weiss. "Quantification of Mutant Huntingtin Protein in Cerebrospinal Fluid from Huntington's Disease Patients." Journal of Clinical Investigation, vol. 125, no. 5, 2015, pp. 1979–1986., doi:10.1172/jci80743.

37. Zhang, Ying, Jeffrey D. Long, James A. Mills, John H. Warner, Wenjing Lu, and Jane S. Paulsen. "Indexing Disease Progression at Study Entry with Individuals At-risk for Huntington Disease." American Journal of Medical Genetics Part B: Neuropsychiatric Genetics 156.7 (2011): 751-63. Print.

38. Zhao, Ting, Yan Hong, Xiao-Jiang Li, and Shi-Hua Li. "Subcellular Clearance and Accumulation of Huntington Disease Protein: A Mini-Review." Front Mol Neurosci. 9, 27 (2016).