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UNIVERSITY OF CALIFORNIA SAN DIEGO

Development of Salivary Inflammatory Biomarkers for Disease Progression

in Huntington's Disease

A Thesis submitted in partial satisfaction of the

requirements for the degree of

Master of Science

in

Biology

by

Aeri Kim

Committee in Charge:

    Professor Jody Corey-Bloom, Chair
    Professor James Kadonaga, Co-Chair
    Professor Michael David

2018

The Thesis of Aeri Kim 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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ABSTRACT OF THE THESIS

Development of Salivary Inflammatory Biomarkers for Disease Progression

in Huntington's Disease

by

Aeri Kim

Master of Science in Biology

University of California San Diego, 2018

Professor Jody Corey-Bloom, Chair
Professor James Kadonaga, Co-Chair

Huntington's disease (HD) is an inherited, neurodegenerative disease characterized by cognitive, psychiatric, and motoric dysfunction, in which the timing and rate of disease progression can be difficult to assess. Recently, inflammatory molecules have been studied for various neurodegenerative diseases, such as Alzheimer's disease, Amyotrophic Lateral Sclerosis, and Parkinson's disease, due to their role in neurodegeneration and immune system activation. In this study, we examined the feasibility of quantifying levels of cortisol, C-reactive protein, interleukin 6, and interleukin 1β in saliva. Next, we correlated these inflammatory biomarker levels in saliva with clinical measures. Lastly, we attempted to correlate salivary levels of the inflammatory biomarkers with levels found in plasma.

The results from this study show that measurement of inflammatory markers in saliva offers significant promise as relevant, non-invasive disease biomarkers for HD. Saliva samples can be collected efficiently and safely by minimally trained personnel, enabling frequent relatively inexpensive collections. Significant correlations between salivary inflammatory levels and measures of cognitive, motor, and functional ability indicate its relevance to the clinical state of the HD patient and offer promise for both clinical research and therapeutic treatment trials.

Introduction

Huntington's disease (HD) is a progressive, neurodegenerative disease caused by an expanded trinucleotide CAG repeat in the huntingtin gene (htt) on chromosome 4p that is inherited by autosomal dominance (Ross and Tabrizi, 2011). This unstable, trinucleotide expansion confers a toxic gain of function that leads to abnormal aggregation of mutant huntingtin protein in cells, resulting in selective neuronal death in striatum and cerebral cortex that is later observed to affect the entire brain (Walker, 2007). HD manifests in cognitive, motoric, and psychiatric symptoms, with no known treatment (Leavitt, Weir, and Sturrock, 2011). Additionally, the disease has been reported to have a negative, high correlation with age of onset, and positive correlation with CAG repeat length and disease severity—including functional impairment (Ross, Brandt, Bylsma, Gross, Stine, and Ranen, 1996).

Genetic testing may easily identify patients with the HD mutation who are certain to progress down a specific clinical course; however, determination of the timing of onset and disease progression for individual patients continues to be extremely difficult. Therefore, the identification of novel biomarkers that can objectively determine symptom onset and can track progression will greatly assist clinicians in the treatment and support of patients with, and at risk for, HD.

Neuroinflammation is pivotal to the onset and progression of many neurological and neurodegenerative disorders. There is increasing evidence that sustained activation of

inflammatory neural cells, such as microglia, contributes to disease progression (Glass, et al., 2010; Brites and Vaz, 2014; Cartier, et al., 2014). With tissue damage, microglia are activated and begin to produce a plethora of cytokines and inflammatory proteins to initiate tissue repair (Möller, 2010). Typically, this response is resolved once the damaged tissue is repaired and the debris is removed. Because neurodegenerative diseases are a result of continuous neuronal damage and death, this immune and inflammatory response is sustained. One particular method by which neurodegeneration occurs is by protein aggregation – as is the case in Alzheimer's disease, spinocerebellar ataxias, and HD – which the body considers to be foreign or hazardous to the cell. With the chronic accumulation of protein aggregates, microglia are constantly activated to produce proinflammatory cytokines.

Thus, inflammatory markers have been extensively studied in blood and cerebrospinal fluid (CSF) with regard to neurodegenerative diseases, notably Alzheimer's Disease (AD), Parkinson's Disease (PD), Amyotrophic Lateral Sclerosis (ALS) and, more recently, HD (Chang, et al., 2014; Sanchez-Lopez, et al., 2012; Leavitt, Weir, Sturrock, 2011). It has been suggested that inflammatory changes in the brain may play a significant role in the pathogenesis and neurotoxicity of HD, thus signifying the potential for inflammatory markers as promising biomarkers in HD progression (Chang, et al., 2014).

Cortisol, infamously known as the "stress hormone," is a glucocorticoid that is produced as a response to stress and regulates a spectrum of pathways from metabolism to immune response. Cortisol secretion is regulated by the hypothalamic-pituitary-

adrenocortical axis (HPA), and has been shown to be elevated during psychosocial stress (Dickerson and Kemeny, 2004; Heuser, et al., 2006). However, chronic cortisol secretion and elevated levels of this glucocorticoid lead to adverse effects on the body – some leading to disease, like Cushing's disease (Pivonello, et al., 2015; Raff and Carrol, 2015; Joseph and Golden, 2017). Additionally, neurodegenerative diseases like Alzheimer's and Parkinson's diseases have repeatedly been shown to have elevated levels of cortisol in plasma compared to normal, age-matched controls (Hartmann, et al., 1997; Weiner, et al., 2015). Because HD is hereditary, gene carriers who have not yet manifested symptoms associated with HD experience anxiety, depression, and irritability (Klöppel, et al, 2010). Therefore, levels of cortisol may help in monitoring those who are premanifest subjects and those who are slowly beginning to manifest symptoms.

C-reactive protein (CRP) is seen to be increased in plasma in individuals with neurodegenerative diseases, especially in premanifest HD patients (Wang, et al., 2014; Leavitt, Weir, Sturrock, 2011). Increased oxidative stress due to cellular dysfunction and impaired metabolisms may support its pathogenic role in HD.

Interleukin 6 (IL-6) is a conventional four-helix bundle responsible for the final differentiation of B cells into antibody-producing cells, and is an inflammatory marker of neuronal cell death (Tadamitsu, 1989). In addition to its role in B cell differentiation, IL-6 has a major role in controlling inflammation as both a pro-inflammatory and anti-inflammatory agent. As neurodegeneration leads to neuronal tissue death, activating the immune system, IL-6 becomes a key player in inducing or suppressing inflammatory reactions to cell death.

Thus, it is of no surprise that there are elevated levels of IL-6 cytokines in Alzheimer's disease, Parkinson's disease, and HD, seen directly in the diseased brain cortices or circulating through the plasma and cerebrospinal fluid (CSF) (Strauss, et al., 1992; Wood, et al., 1993; Nagatsu and Sawada, 2007; Mogi, et al., 1996; Björkqvist, 2008).

Another biomarker of interest is interleukin 1β (IL-1β), a potent pro-inflammatory cytokine (Sager, et al., 2016). In addition to its key role in immune activation and inflammation, IL-1β is produced by microglia to regulate sleep, memory, and synaptic plasticity (Pang, et al., 2012). Similar to cortisol and other inflammatory cytokines, chronic production of IL-1β at elevated levels results in tissue injury – neurons are particularly sensitive, and this neuronal damage serves as a marker when monitoring the development and progression of neurodegenerative diseases (Rothwell, 2003; Wang, et al., 2008; Lull and Block, 2010; Pang, et al., 2012). Of interest, IL-1β has been tied to Alzheimer's disease because of a positive correlation with amyloid burden (Halle et al., 2008; Heneka et al., 2012; Ghosh, et al., 2013). In fact, polymorphisms within the gene responsible for IL-1β have been shown to be associated with stronger risk for Alzheimer's disease (Rothwell, 2000).

While blood is the most common biofluid collected in clinical settings, saliva provides an easier, less invasive form of sample collection that is cheaper and requires no specialized training by study personnel. In addition, saliva is safer as it is less likely to transmit diseases through contact or needle-stick injury and the molecular components that make up the sample do not undergo much biomolecular change (Prasad, et al., 2016). Saliva has been used as a discerning biosample for decades--from measuring levels of cotinine for

environmental tobacco smoke by nonsmokers as early as the 1990s (Benowitz, 1996) to

exploiting the metabolome and studying metabolites as potential medical biomarkers during

the last five years (Zhang, Sun, Wang, 2012). Indeed, salivary cortisol measurement has been

widely accepted, and is thought to be a reliable mirror of the hypothalamic-pituitary axis

and more physiologically relevant than plasma since the stress and anxiety of venipuncture

can induce the iatrogenic increase of cortisol (Kirschbaum, et al. 2008; Vining, et al., 1983;

Chiappin, et al., 2007). Today, virtually the entire proteome of human saliva has been

established and identified to be potential diagnostic markers for a variety of diseases (Ruhl,

2012).

Saliva, itself, is a fluid produced in the mouth. It has many functions, mostly

promoting oral health by maintaining proper pH levels and bacterial growth (Chiappin, et al.,

2007; Wong, et al., 2017). The α-amylase and other enzymes in saliva help to break down

food before subsequent digestion in the stomach. Saliva is produced from three major pairs

of salivary glands (parotid, sublingual, and submandibular) and an abundance of minor

salivary glands (Holmberg and Hoffman, 2014). The anatomical structures of the major

salivary glands are essentially the same – consisting of a duct that opens up to the oral

cavity. The acinar cells at the ends of these secretary ducts are what produces the saliva,

which becomes the product of a myriad of environments. The acini are "surrounded by an

extracellular matrix, myoepithelial cells, myofibroblasts, immune cells, endothelial cells,

stromal cells, and nerve fibers (Holmberg and Hoffman, 2014). In addition, the major

salivary glands are greatly vascularized by the facial and temporal arteries. There are three

major pathways through which compounds are passed from the plasma into the saliva: 1) ultrafiltration through gap junctions between cells; this allows for extremely small molecules like ions, water, steroids to pass through, 2) transudation of canonical plasmatic molecules like albumin or leukocytes via crevicular fluid or from the oral mucosa, and 3) selective transport (active transport and passive diffusion) through cellular membranes. Cytokines and leukocytes are primarily cleared from the periphery to saliva through crevicular fluid transudation (Chiappin, et al., 2007; Teles, et al., 2009).

In this study, we sought to determine if cortisol, CRP, IL-6, and IL-1β could be identified and reliably measured in saliva and plasma from premanifest HD, manifest HD, and normal control subjects. In addition, we aimed to relate the levels of these four inflammatory biomarkers in saliva to the levels found in plasma, and examine whether these levels correlate meaningfully with clinical measures. The advent of an effective, dependable salivary biomarker would meet the urgent need for a less invasive means of identifying and monitoring HD disease progression.

Subjects and Methods

1.1 Regulatory Documents

This study was approved by the University of California San Diego Institutional

Review Board in accordance with the requirements of the Code of Federal Regulations on

the Protection of Human Subjects. Informed consent was obtained from all participants

prior to study start. All necessary regulatory documents were maintained in the study file,

which includes the subject identification log, screening/enrollment log, and all signed

informed consent forms.

2.1 Participants

A total of 122 subjects, including 35 manifest HD patients, 46 premanifest HD

patients, and 41 normal controls, were recruited through the UCSD Huntington's disease

Society of America (HDSA) Center of Excellence (CoE) – directed by Dr. Jody Corey-Bloom.

Demographics were collected on all subjects including age, gender, weight, years of

education, CAG repeat length, and family history. Each participant provided saliva and blood

samples within six months of their clinical assessment testing date. In addition to

demographic information collection, each participant participated in clinical assessments, as

described below.

3.1 Clinical Assessments

All study participants were subjected to clinical assessments, including cognitive testing, behavioral and functional measures, and motor ratings.

3.1.1 Cognitive Assessments

The cognitive testing comprised brief tests of language, attention, problem-solving, visuospatial abilities, and memory. The cognitive battery included the Mini-Mental State Examination (MMSE), Montreal Cognitive Assessment (MoCA; Nasreddine et al., 2005), and Symbol Digit Modalities (SDM) test. The MMSE is a 30-item questionnaire that assesses cognitive impairment. It consists of six categories: orientation, registration, attention and calculation, recall, language, and complex commands. Each item is worth 1 point, and the participant is awarded a score out of 30. The MoCA examines cognitive impairment through memory recall, visuospatial abilities, executive function, attention, language, and orientation. The maximum total of points available is 30, and a point is deducted for every task that is incomplete or incorrect. The Symbol Digit Modalities test is a neuropsychological test that assesses processing speed and the ability to correctly pair a symbol to its appropriate digit. The legend on the top of the sheet consists of nine digits (1-9) with their respective symbols. The legend is followed by a list of digits, where the participant is then asked to fill in the boxes below each digit with its corresponding symbol quickly and accurately. The test is timed for 90 seconds, and the maximum total of points available is 110 points.

3.1.2 Behavioral and Functional Assessments

Behavioral and psychiatric changes were assessed using the short form Problem Behaviors Assessment (PBA-s) and the Hospital Anxiety and Depression Scale/Snaith Irritability Scale (HADS-SIS). 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. 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 indicating more behavioral issues.

Functional proficiency was evaluated using the UHDRS Total Functional Capacity (TFC). The TFC assesses the participant's ability to 1) engage satisfactorily in gainful employment or voluntary work, 2) engage in personal and family finances, 3) carry out routine domestic tasks, 4) successfully perform activities of daily living, such as eating, dressing, and bathing, in addition to 5) the care environment that is most appropriate, whether this be at home, with chronic care, or in a full-time skilled nursing facility. The maximum possible score is 13, signifying that the participant is able to fully care for themselves and fulfill the tasks indicated independently. Points are deducted for any loss of capacity to independently fulfill these tasks.

3.1.3   Motoric Assessments

The UHDRS Total Motor Score is a motoric assessment composed of 16 sections using a rating scale of 0 (no abnormality present) to 4 (motor abnormality severely present). The 16 sections are ocular pursuit (horizontal and vertical), saccade initiation (horizontal and vertical), saccade velocity (horizontal and vertical), dysarthria, tongue protrusion, finger taps (right and left), pronate/supinate hand motion (right and left), the Luria, arm rigidity (right and left), body bradykinesia, maximal dystonia (trunk, RUE, LUE, RLE, LLE), maximal chorea (face, BOL, trunk, RUE, LUE, RLE, LLE), gait, tandem walking, retropulsion pull test, and the administrator's diagnostic confidence level. 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), while the sum of all maximal chorea sub-scores, maximum score of 28 points, comprises the Total Chorea Score (TCS). The certified neurologist, Dr. Corey-Bloom, was responsible for administering and rating the patients on the UHDRS TMS. In addition, the Timed Up and Go (TUG) was utilized to assess for mobility as subjects walked around a cone that was ten feet away and then returned to the starting position. Manifest HD subjects with more balance and gait problems would be expected to take longer to complete this task compared to normal controls.

3.1.4   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; with higher scores indicating 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 length. The CAP score (Zhang, 2011), which is computed as $CAP = Age_0 \times (CAG-33.7)$, 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.

4.1   Saliva Sample Collection and Preparation

Participants were asked if they had consumed any alcohol, caffeine, nicotine, and/or had taken any medications within the last twelve hours of their visits, in addition to whether they engaged in any weekly vigorous exercise. Then, they were asked to rinse their mouths thoroughly with water for ten seconds to minimize any acidic or high sugar foods that can compromise the pH and bacterial growth in the mouth. After rinsing, the participants were prohibited from any food or liquid consumption for thirty minutes, and saliva samples were collected thereafter. Saliva is collected into a standard 10 mL tube via unstimulated passive drool method. Each sample collection was aliquoted into 0.5 mL aliquots and stored at -80°C until they were assayed. At the time of use, saliva fractions were thawed and centrifuged at 10,000 xg for ten (10) minutes at 4°C to remove any

insoluble components and cellular debris. The supernatants were collected and used for all assays.

5.1   Plasma Sample Collection and Preparation

A certified phlebotomist drew four (4) mL of whole blood from each participant. The blood was immediately taken to the lab and spun at 4,500 RPM for ten (10) minutes at room temperature using a Thermo Scientific Sorvall ST 16 benchtop centrifuge. After centrifugation, the plasma component was aliquoted into 0.5 mL aliquots and stored at -80°C until they were assayed. 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.

6.1   Enzyme-Linked Immunosorbent Assays (ELISAs)

Saliva and plasma samples were assayed for levels of cortisol, CRP, IL-6, IL-1β, and α-amylase, which acted as an additional positive control due to its established and abundant presence in saliva.

6.1.1   Saliva

All saliva samples were run by Salimetrics, LLC in Carlsbad, California. Commercial ELISA kits were used to assay for levels of cortisol, CRP, IL-6, IL-1β, and α-amylase.

6.1.2 Plasma

Plasma samples for human CRP, IL-6, IL-1β were assayed by Dr. Elizabeth Thomas at the Scripps Research Institute (human CRP commercial ELISA kit purchased from R & D Systems, human IL-6 and human IL-1β commercial ELISA kits purchased from Thermo Fisher Scientific).

7.1 Analysis and Statistical Methods

All data are expressed as mean ± standard deviation unless otherwise indicated. Outliers were identified and removed using the iterative Grubbs test function in GraphPad Prism v6.0 for MacOSX. All comparisons of variables to determine significance of differences were done using IBM's SPSS Statistics software. Comparisons between two groups were made using Independent Samples T-test; comparisons among the three diagnostic groups were done using an Analysis of Variance (ANOVA) or Analysis of Covariance (ANCOVA) test, controlling for age, weight, and years of education. Overall ANOVAs were performed, and post-hoc analyses were run using Tukey's test. $p < 0.05$ was considered significant. Partial correlations, controlling for age, weight, and years of education, were calculated to consider any relationship between inflammatory biomarker levels and clinical measures (which were not normally distributed).

Results

Demographic and Clinical Characteristics Reflect HD Symptoms. Demographic information for

the Inflammatory biomarker cohort is provided in Table 1. Among the final 122 participants,

the normal control group consisted of 35 subjects, the premanifest HD group consisted of

46 subjects, and the manifest HD group consisted of 41 subjects. There were about the

same number of males and females in the normal control group, but more females in both

the premanifest HD group (26 females to 20 males) and manifest HD group (26 females to

15 males). Not surprisingly, manifest HD patients (56.95 ± 12.28 years) were older than

premanifest HD subjects (43.30 ± 13.71 years; p=0.000*) and had higher mean CAG repeat

lengths (42.93 ± 2.73 and 41.68 ± 2.75, respectively; p=0.036). Manifest HD patients also

had fewer years of education (14.22 ± 3.75 years) than both premanifest HD subjects (16.24

± 3.13 years; p=0.005) and normal controls (15.40 ± 2.78 years; p=0.118). In addition, HD

patients weighed significantly less (149.83 ± 36.46 pounds) than their premanifest (172.90 ±

46.16 pounds; p=0.013) and normal control counterparts (171.94 ± 44.12 pounds; p=0.027),

a metabolic hallmark of Huntington's disease.

The clinical characteristics of our subjects are shown in Table 2. Manifest HD patients

had significantly lower mean MMSE (24.83 ± 3.18 points) and MoCA (22.68 ± 4.12 points)

scores, suggestive of greater cognitive dysfunction, than premanifest HD participants (28.17

± 1.54; p=0.000 and 27.24 ± 2.34 points, respectively; p=0.000) and normal controls (28.56

± 1.04; p=0.000 and 27.19 ± 1.63 points, respectively; p=0.000). Further, the manifest HD

patients scored significantly lower on the SDM test (24.12 ± 9.15 points) than premanifest

HD subjects (51.28 ± 10.45 points; p=0.000) and normal controls (49.37 ± 9.14 points;

p=0.000). As expected, manifest HD patients had significantly reduced functional abilities,

with a mean TFC score of 8.57 ± 2.71, compared to premanifest HD participants (12.85 ±

0.56; p=0.000). Psychiatric and behavioral assessments showed manifest HD patients scored

higher (PBA-s = 14.73 ± 15.06, HADS-SIS = 22.84 ± 15.88) than premanifest HD subjects

(PBA-s = 5.65 ± 8.65; p=0.000 and HADS-SIS = 17.58 ± 13.39; p=0.009). Also, manifest HD

patients scored higher on motor assessments (34.62 ± 16.81) and took much longer to

complete the TUG (12.09 ± 3.20 seconds) than premanifest HD subjects (2.81 ± 2.69,

p=0.000; 9.39 ± 1.53 seconds, p=0.000, respectively).

Table 1. Demographic Characteristics for Subject Cohorts (N=122). All demographic information obtained prior to study and sample collection start. **Abbreviations**: DBS = Disease Burden Score; AofO = Age of Onset; PAO = Parental Age of Onset. Values are represented as mean (range) except for Gender.

Table 1. Demographic Characteristics for Subject Cohorts (N = 122)				Overall
	NC	**PM**	**HD**	**p-value**
n	35	46	41	
Gender, F:M	17:18	26:20	26:15	
Age, yrs	57.74 (23 - 78)	43.30 (19 - 71)‡‡	56.95 (30 - 76)ºº	0.000
Weight, lbs	171.94 (106 - 306)	172.90 (99 - 290)	149.83 (90 - 263)*º	0.024
Education	15.40 (12 - 22)	16.24 (12 - 22)	14.22 (5 - 24)ºº	0.018
CAG Repeats		41.68 (38 - 51)	42.93 (37 - 49)º	0.036
DBS		248.39 (112 - 368)	388.14 (273 - 578)ºº	0.000
AofO, yrs			50.63 (22 - 68)	/
PAO, yrs		52.21 (24 - 75)	48.41 (27 - 70)	0.222

*, P<0.05 between NC and HD; **, P<0.01 between NC and HD.
º, P<0.05 between PM and HD; ºº, P<0.01 between PM and HD.
‡, P<0.05 between NC and PM; ‡‡, P<0.01 between NC and PM.

Table 2. Clinical Characteristics for Subject Cohorts. **Abbreviations**: UHDRS = Unified Huntington's Disease Rating Scale; MMSE = Mini Mental State Exam; MoCA = Montreal Cognitive Assessment; SDM = Symbol Digit Modalities Test; TFC = Total Functional Capacity; PBA-s = Problem Behaviour Assessment-short form; HADS-SIS = Hospital Anxiety and Depression Scale – Snaith Irritability Scale; TMS = Total Motor Score; TUG = Timed Up and Go. Values are represented as mean (range).

Table 2. Clinical Characteristics for Subject Cohorts, mean (range)				Overall p-value
	NC	PM	HD	
MMSE	28.56 (26 - 30)	28.17 (25 - 30)	24.83 (19 - 30)ºº**	0.000
MoCA	27.19 (22 - 30)	27.24 (20 - 30)	22.68 (13 - 30)ºº**	0.000
SDM	49.37 (31 - 68)	51.28 (24 - 67)	24.12 (11 - 47)ºº**	0.000
UHDRS TFC	12.93 (12 - 13)	12.85 (10 - 13)	8.57 (2 - 13)ºº**	0.000
PBA-s	2.84 (0 - 12)	5.65 (0 - 33)	14.73 (0 - 56)ºº**	0.000
HADS-SIS	13.36 (0 - 31)	17.58 (1 - 53)	22.84 (0 - 64)ºº**	0.000
TMS	1.54 (0 - 10)	2.81 (0 - 11)	34.62 (9 - 70)ºº**	0.000
Total Chorea	/	0.19 (0 - 2)	6.93 (1 - 17)ºº	0.000
TUG, sec	9.21 (7 - 13)	9.39 (7 - 13)	12.09 (9 - 23)ºº**	0.000

*, P<0.05 between NC and HD; **, P<0.01 between NC and HD.
º, P<0.05 between PM and HD; ºº, P<0.01 between PM and HD.

Salivary inflammatory biomarkers are present in human saliva and can be quantified by ELISA.
Levels of cortisol, CRP, IL-6, IL-1β were quantifiable in human saliva by using an Enzyme-Linked Immunosorbent Assay.

Salivary inflammatory biomarkers distinguish different diagnostic groups. We first sought to determine whether levels were different according to different diagnostic groups (see Table 3).

Cortisol

Levels of salivary cortisol were significantly higher in manifest HD patients (0.17 ± 0.08 μg/dL) compared to premanifest individuals (0.12 ± 0.06 μg/dL) (p=0.047) (Figure 1A).

C-reactive protein

Levels of salivary CRP were significantly higher in premanifest participants (3493.29 ± 3738 pg/mL) compared to normal controls (2400.24 ± 1745 pg/mL; p=0.032) (Figure 1B).

Interleukin 6

Levels of salivary IL-6 were significantly higher in manifest HD patients (45.73 ± 47 pg/mL) compared to both premanifest individuals (13.36 ± 13 pg/mL, p=0.048) and normal controls (19.43 ± 19 pg/mL; p=0.024) (Figure 1C).

Interleukin 1β

Levels of IL-1β were significantly higher in manifest HD patients (873.14 ± 803 pg/mL) compared to premanifest individuals (389.94 ± 261 pg/mL; p=0.021) (Figure 1D).

Table 3. Inflammatory Biomarker Levels in Saliva. **Abbreviations**: CRP = C-Reactive Protein; IL-6 = Interleukin 6; IL-β = Interleukin 1-βeta. Values are represented as mean (range).

Table 3. Inflammatory Biomarker Levels in Saliva, mean (range)				Overall p-value
	NC	**PM**	**HD**	
Cortisol, µg/dL	0.13 (0.02 - 0.31)	0.12 (0.03 - 0.26)	0.17 (0.07 - 0.34)º	0.132
n	19	16	18	
CRP, pg/mL	2400.24 (325 - 7075)	3493.29 (292 - 14333)+	2981.00 (530 - 10713)	0.098
n	29	42	31	
IL-6, pg/mL	19.43 (3.65 - 63.17)	13.36 (1.35 - 56.10)	45.73 (0.84 - 175.17)*º	0.043
n	14	21	17	
IL-1β, pg/mL	622.90 (107 - 1521)	389.94 (32 - 1058)	873.14 (107 - 2825)º	0.036
n	15	27	21	
Amylase, U/mL	163.62 (13 - 390)	140.64 (10 - 385)	137.80 (27 - 385)	0.439
n	33	44	39	

*, P<0.05 between NC and HD; **, P<0.01 between NC and HD.

º, P<0.05 between PM and HD; ºº, P<0.01 between PM and HD.

+, P<0.05 between NC and PM; ++, P<0.01 between NC and PM.
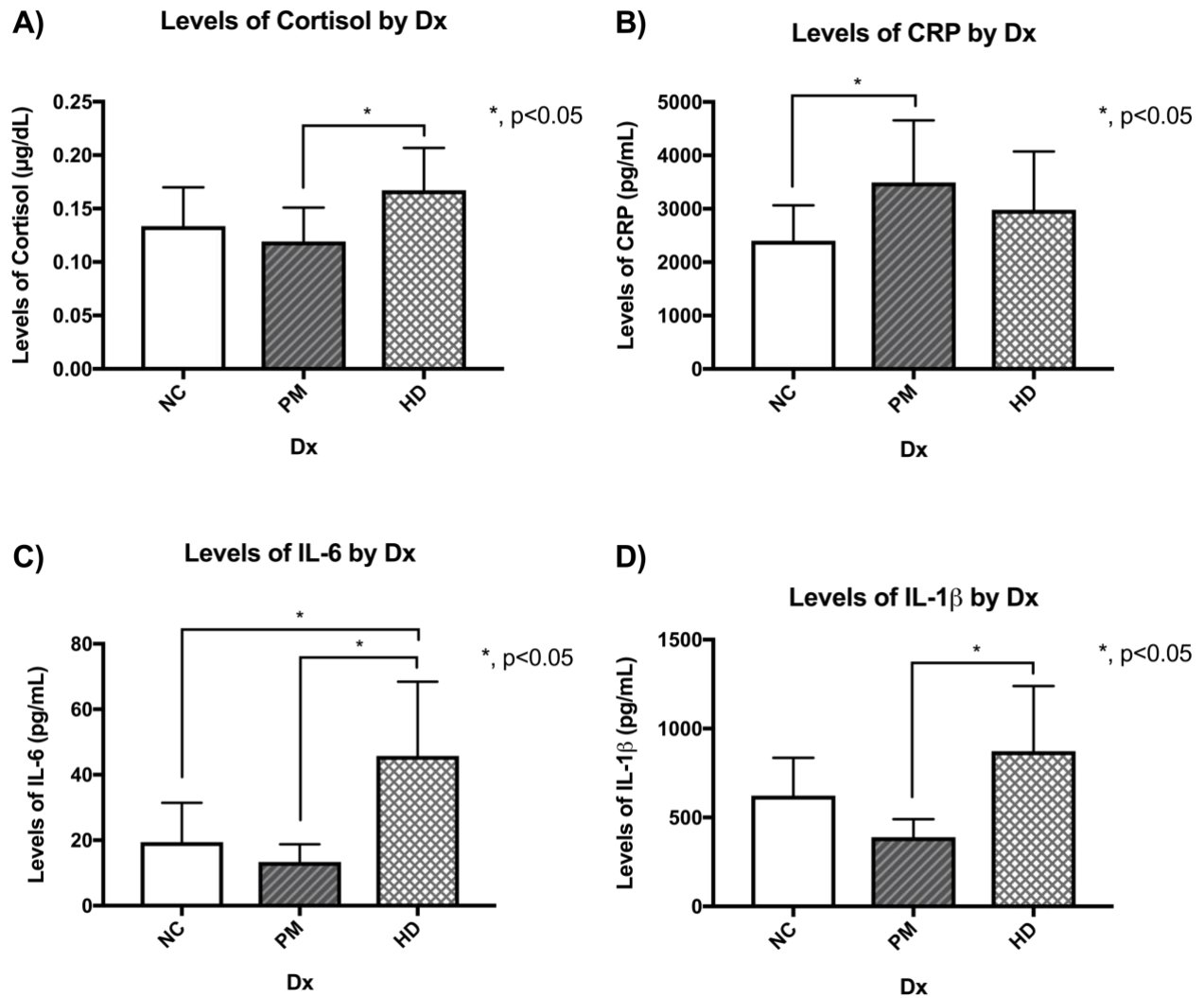
Figure 1. Levels of inflammatory markers (cortisol, CRP, IL-6, IL-1β) by diagnoses (NC, PM, HD). **A)** Cortisol levels (ug/dL) increased in HD patients compared to premanifest subjects. **B)** CRP (pg/mL) is elevated in premanifest subjects compared to normal controls. **C)** HD patients have higher levels of IL-6 (pg/mL) than both premanifest subjects and normal controls. **D)** IL-1β (pg/mL) increased in HD patients compared to premanifest subjects. Each bar is represented as mean with 95% CI.

Salivary inflammatory biomarkers correlate with clinical measures in gene positive

participants. Next, we examined whether levels of the four salivary markers correlated with

our clinical measures. In a cohort consisting of both premanifest and manifest HD subjects,

there was a moderate correlation between salivary IL-6 levels and DBS (R=0.507, p=0.001)

(Figure 2A), and a moderate correlation between salivary IL-6 levels and TFC (R= -0.337,

p=0.033) (Figure 2B). Salivary IL-1β levels had similar correlations with disease burden

(R=0.305, p=0.039) (Figure 3A) and TFC (R= -0.358, p=0.014) (Figure 3B). Salivary IL-1β also

showed a moderate correlation with TMS (R=0.436, p=0.002) (Figure 3C).
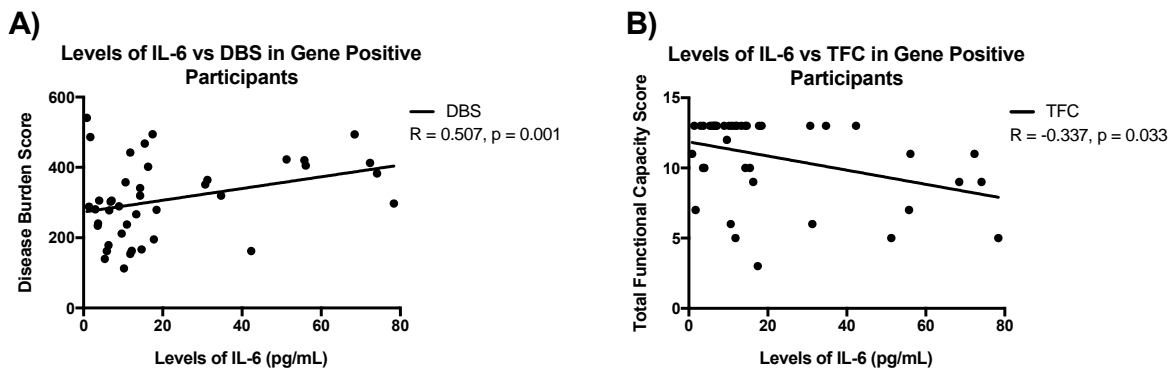

Figure 2. Partial correlations between levels of salivary IL-6 by clinical measures. **A)** There is a strong, statistically significant correlation between salivary IL-6 levels and disease burden score (R = 0.507, p = 0.001). **B)** There is a modest, statistically significant correlation between salivary IL-6 levels and total functional capacity score (R = -0.337, p = 0.033).
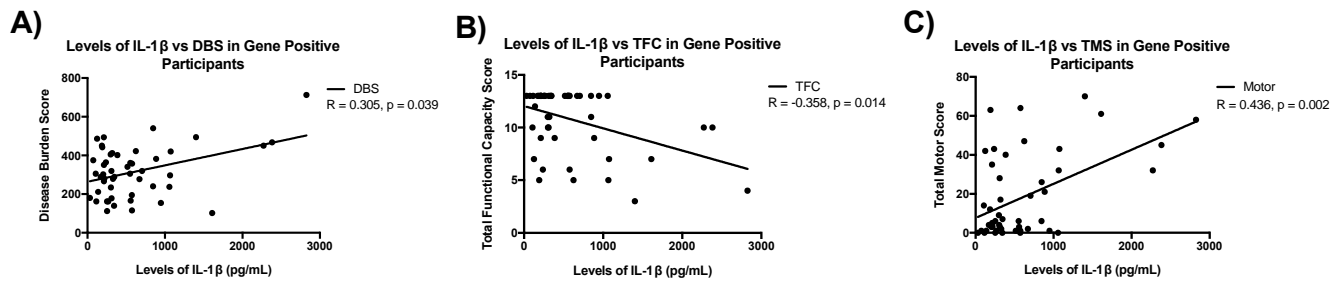
Figure 3. Partial correlations between levels of salivary IL-1β by clinical measures. **A)** There is a modest, statistically significant correlation between salivary IL-1β levels and disease burden score (R = 0.305, p = 0.039). **B)** There is a modest, statistically significant correlation between salivary IL-1β levels and total functional capacity score (R = -0.358, p = 0.014). **C)** There is a moderate, statistically significant correlation between salivary IL-1β levels and total motor score (R = 0.436, p = 0.002).

Plasma inflammatory biomarkers distinguish different diagnostic groups. We sought to determine whether inflammatory biomarker levels were different according to different diagnostic groups in plasma. Results are shown in Table 4.

C-reactive protein

Levels of plasma CRP were higher in premanifest participants (22.10 ± 19.82 ng/mL) compared to normal controls (12.37 ± 12.95 ng/mL; NS) and manifest HD patients (11.45 ± 18.00 ng/mL; NS) (Figure 4A).

Interleukin 6

Levels of plasma IL-6 were significantly higher in manifest HD patients (1.09 ± 1.19 pg/mL) than premanifest HD subjects (0.22 ± 0.18 pg/mL; p=0.018) (Figure 4B).

Interleukin 1β

Levels of plasma IL-1β were higher in premanifest HD subjects (0.52 ± 0.67 pg/mL) than manifest HD subjects (0.17 ± 0.13 pg/mL; NS) (Figure 4C).

Table 4. Inflammatory Biomarker Levels in Plasma. **Abbreviations**: CRP = C-Reactive Protein; IL-6 = Interleukin 6; IL-1β = Interleukin 1-βeta. Values are represented as mean (range).

Table 4. Inflammatory Biomarker Levels in Plasma, mean (range)				Overall p-value
	NC	**PM**	**HD**	
CRP, ng/mL	12.37 (0.83 - 38.20)	22.10 (3.86 - 65.72)	11.45 (0.27 - 75.72)	0.120
n	14	24	17	
IL-6, pg/mL	0.42 (0.08 - 0.7)	0.22 (0.01 - 0.593)	1.09 (0.01 - 3.06)º	0.046
n	5	10	6	
IL-1β, pg/mL	0.69 (0.03 - 0.87)	0.52 (0.05 - 2.38)	0.17 (0.01 - 0.33)	0.339
n	8	11	7	

º, P<0.05 between PM and HD

Correlations between salivary and plasma inflammatory biomarkers. There was only a weak correlation between salivary and plasma CRP levels (n=46; R=0.203; p=0.192) (Figure 5A). There was, however, a strong positive correlation between saliva and plasma for IL-6 (n=16; R=0.879; p<0.001) (Figure 5B) and a moderate positive correlation between saliva and plasma (n=23; R=0.501; p=0.024) (Figure 5C) for IL-1β.

A) Levels of CRP in Plasma by Dx

B) Levels of IL-6 in Plasma by Dx

C) IL-1B Plasma Levels in Dx
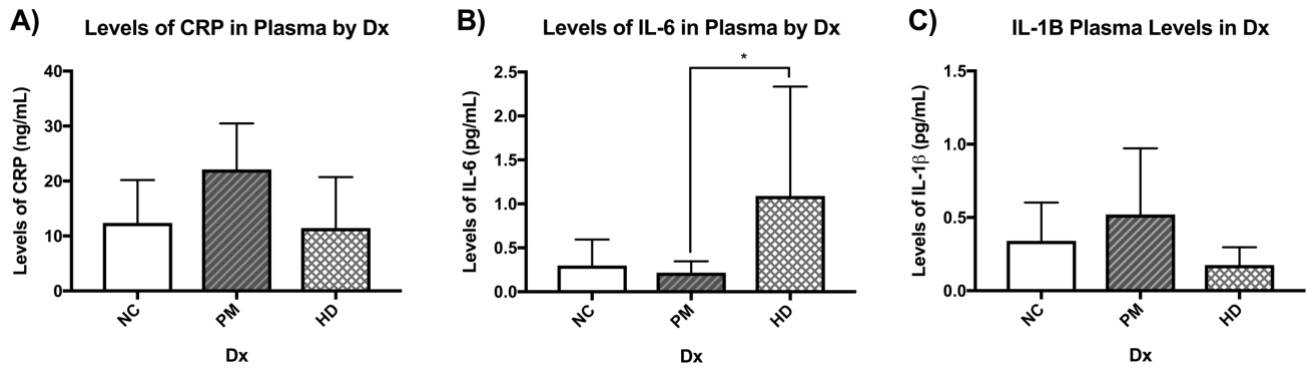
Figure 4. Plasma levels of inflammatory markers (CRP, IL-6, IL-1β) by diagnoses (NC, PM, HD). **A)** CRP (pg/mL) is elevated in premanifest subjects compared to normal controls. **B)** HD patients have higher levels of IL-6 (pg/ML) than both premanifest HD subjects and normal controls. **C)** IL-1β (pg/mL) increased in premanifest HD subjects compared to manifest HD patients. Each bar is represented as mean with 95% CI.
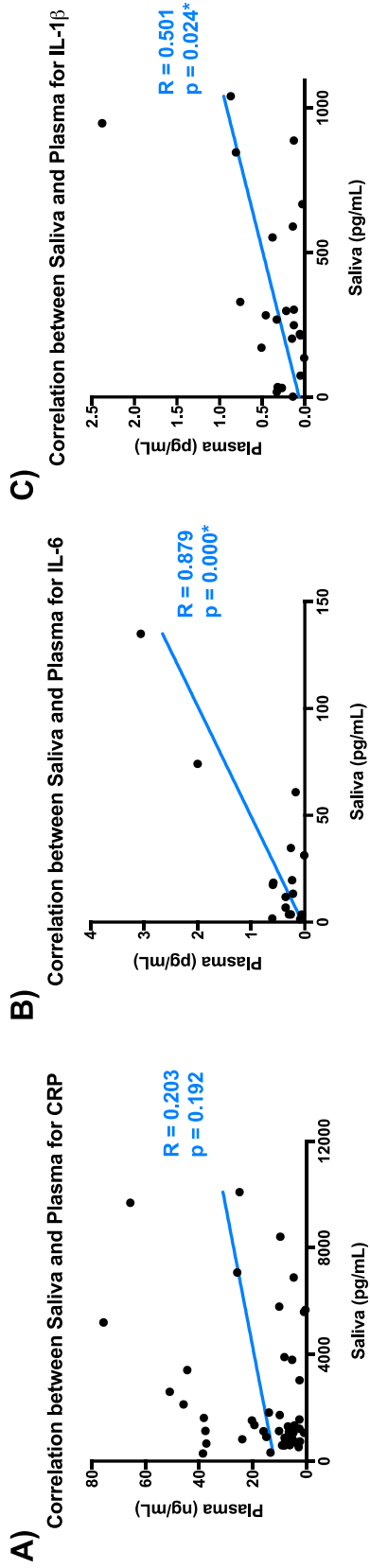
Figure 5. Partial correlations between saliva and plasma for C-reactive protein (CRP), interleukin 6, (IL-6), and interleukin 1β (IL-1β). **A)** There is a modest positive correlation (R = 0.203) and no statistically significant effect (p = 0.192) between saliva and plasma in CRP. **B)** There is a strong positive correlation (R = 0.879) with a statistically significant effect (p < 0.001) for IL-6. **C)** There is a moderate positive correlation (R = 0.501) with a statistically significant effect (p = 0.024) for IL-1β.

Discussion

The need for reliable, state and stage biomarkers in HD patients is great, due to the immense variability in symptom onset and unpredictable severity of disease progression. Although there is no cure for HD to date, effective treatment is on the horizon. Having the ability to determine when patients are close to phenoconversion is important to ensure that patients enroll in clinical treatment trials at specific disease stages and get appropriate early treatment when it becomes available. Neuroinflammation has been shown to exacerbate neurodegeneration, due to the presence of numerous pro-inflammatory cytokines and proteins caused by the sustained activation of microglia (Tai, et al., 2007; Sapp, et al., 2001; Pavese, et al., 2006; Björkqvist, et al., 2008). Indeed, as seen with Parkinson's disease, by the time a patient is formally diagnosed as manifest HD, there is very little of the basal ganglia and cortex that remains intact (Reiner, et al., 2015). Thus, we believe that looking at inflammatory markers will be useful for monitoring HD symptom onset and tracking disease progression.

In the current study, we recruited and obtained demographic information, clinical measures, and biosamples from 122 participants, including 41 manifest HD, 46 premanifest HD, and 35 NC.

Importantly, we determined that inflammatory measures, including cortisol, CRP, IL-6, and IL-1β, are clearly present in saliva, which is extremely promising as saliva can be collected noninvasively and does not require skilled personnel to handle it. Since whole saliva collection is noninvasive, easier to handle, and significantly lower in cost for collection and storage, this method is very appealing and also very applicable for measuring biomarker levels in infants, children, elderly, and conditions where blood and urine sampling may be less feasible (Ruhl, 2012; Chiappin, et al., 2007). Furthermore, the advancement of technology has allowed for mobile, convenient modules that can run a panel analysis with just a single drop of blood, essentially act as a full laboratory. Using saliva as the biofluid will be greatly aid running these tests in locations where sterile environments for blood collection and correct disposal of needles is not possible.

We found that salivary cortisol is elevated in manifest HD patients compared to both normal controls and premanifest HD subjects. This finding is consistent with studies that found increased levels of plasma cortisol in manifest HD patients compared to normal controls and premanifest HD subjects (Adamczak-Ratajczak, et al., 2017; Aziz, et al., 2009; Heuser, et al., 1991; Leblhuber, et al., 1995). Because cortisol is an established marker for stress, we thought that manifest HD patients might have lower levels of cortisol, since HD patients are often quite apathetic. However, the results have consistently shown elevated levels of cortisol in HD patients as compared to normal controls; scientists have largely agreed that this abundance of cortisol is a result of hypothalamic-pituitary adrenal (HPA) axis dysfunction. With the loss of neurons in the hypothalamus (Reed and Neel, 1959; Bruyn

and de Jong, 1973), endocrine pathways that are regulated by the hypothalamus lose their regulatory abilities and go awry.

We also found that salivary CRP is elevated in premanifest HD subjects compared to normal control subjects. This is important because this indicates promise as an early biomarker for disease onset and progression. Clinically, premanifest HD subjects look very much like normal controls – they are prodromal and do not show the classic symptoms of HD. They score just as well or better on functional capacity assessments and neuropsychological tests, and do not have motoric dysfunction. However, the statistically significant difference between these two groups may provide insight into distinguishing one group from the other. This finding is consistent with studies that demonstrated increased levels of plasma CRP in premanifest HD subjects compared to normal controls (Wang, et al., 2014). Interestingly, other groups have shown elevated levels of plasma CRP in manifest or advanced HD patients compared to premanifest or early HD subjects (Stoy, et al., 2005; Sánchez-Lopez, et al., 2013); however, it is unclear whether this increase reflects an acute phase response (Bouwens, et al., 2014) secondary to the use of antipsychotics in the progressed subjects (Gewurz, et al., 1982). Previously, CRP had only been measured in saliva of stress-induced individuals, such as victims of domestic violence and posttraumatic stress disorder (Out, et al., 2012; Fernandez-Botran, et al., 2011; Michopoulos, et al., 2015). To our knowledge, this study is the first to measure CRP in saliva of neurodegenerative disease subjects, and specifically HD.

We found that salivary IL-6 were elevated in HD subjects compared to both normal control and premanifest HD subjects, and salivary IL-1β was elevated in HD subjects compared to premanifest HD subjects. These findings are consistent with studies that demonstrated an increase in plasma IL-6 in early and moderate HD patients compared to normal controls (Björkqvist, et al., 2008; Dalrymple, et al., 2007; Sánchez-Lopez, et al., 2013). At least one study has suggested that plasma IL-6 is increased due to mutant htt in both human HD monocytes and HD murine macrophages and microglia (Björkqvist, et al., 2008). Studies have also shown increased plasma and CSF IL-1β levels in Alzheimer's disease (Fagan and Perrin, 2013), amyotrophic lateral sclerosis (Meissner, et al., 2010), and Parkinson's disease (Mogi, et al., 1994) patients. Previously, IL-6 and IL-1β had only been measured in saliva from stress-induced or psychologically disturbed individuals, such as those with schizophrenia or neuroticism, etc. (La Fratta, 2018; Teles, et al., 2009; McInnis, et al., 2015; Sutin, et al., 2011; Riis, et al., 2015; Chiappelli, et al., 2016). To our knowledge, this study is the first to measure IL-6 and IL-1B in saliva of neurodegenerative disease subjects, and specifically HD.

In the current study, gene positive subjects showed moderate correlations between salivary IL-6 and both DBS and TFC. This moderate negative correlation between salivary IL-6 and TFC is consistent with other studies that have demonstrated similar negative correlations between plasma IL-6 levels and TFC (Chang, et al., 2015). We also found moderate correlations between salivary IL-1β levels and DBS, TFC, and TMS in our gene positive participants. To our knowledge, this study is the first to show a correlation between

IL-1β levels and functional or motoric measures in human patients, although some studies have shown an increase in depression (Levine, et al., 1999; Raison, et al., 2006) and decreased cognitive abilities (Trompet, et al., 2008). There are, however, murine, rat, and piglet models that have shown correlations between IL-1β levels and other clinical measures (Moore, et al., 2009; Anforth, et al., 1998; Jankowsky and Patterson, 1999; Elmore, et al., 2014).

In a small subset of our cohort, preliminary results suggest only a weak correlation between salivary and plasma CRP levels. However, there was a strong positive correlation between saliva and plasma for IL-6 and a moderate positive correlation between saliva and plasma for IL-1β. While there are no other studies of correlations between salivary and plasma CRP, IL-6, and IL-1β in HD patients, there are similar correlations between saliva and plasma CRP levels in depressed adolescents and women who were exposed to partner or domestic violence (Byrne, et al., 2013; Out, et al., 2012). An additional study showed a positive, strong correlation between salivary and plasma IL-6 and IL-1β levels in young men in stressful environments (La Fratta, et al., 2018).

As saliva becomes more promising as a reliable biofluid for examining progression in HD, it will be increasingly important to establish a high correlation between saliva and plasma to better validate salivary assays. One limitation includes the size of our cohorts for saliva-plasma correlations. Like cortisol, future investigations of inflammatory markers will require the collection of more saliva-plasma pairs to contribute toward the effort to establish widely accepted measurements of C-reactive protein, IL-6, and IL-1β. This study

established increased levels of salivary cortisol in, IL-6, and IL-1β in manifest HD patients and increased levels of salivary CRP in premanifest HD subjects. While this confirmed our hypothesis, these four markers are relatively general indicators of immune system activation and inflammatory response. Future work should include investigations of neuron-specific inflammatory biomarkers to reduce the scope to inflammation in the nervous system, as compared to other causes of inflammation, such as arthritis or recent illness.

In conclusion, the results from this study show that measurement of inflammatory markers in saliva offers significant promise as relevant, non-invasive disease biomarkers for HD. Saliva samples can be collected efficiently and safely by minimally trained personnel, enabling frequent relatively inexpensive collections. Significant associations between salivary inflammatory 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 treatment trials.

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Supplementary Material

Table S1. All Analysis of CoVariance tests run while covarying for age, education, and weight. **Abbreviations**: UHDRS = Unified Huntington's Disease Rating Scale; MMSE = Mini Mental State Exam; MoCA = Montreal Cognitive Assessment; SDM = Symbol Digit Modalities test; TFC = Total Functional Capacity; PBA-s = Problem Behaviour Assessment-short form; HADS-SIS = Hospital Anxiety and Depression Scale-Snaith Irritability Scale; TMS = Total Motor Score; TUG = Timed Up and Go; *, $p<0.05$; **, $p<0.01$.

Table S1. ANCOVA Results in Demographic and Clinical Characteristics Among Diagnostic Groups (p-values)			
Parameters	**NC vs PM**	**NC vs HD**	**PM vs HD**
Age, yrs	**0.000***	0.796	**0.000****
Weight, lbs	0.920	**0.027***	**0.013***
Education	0.253	0.118	**0.005****
MMSE	0.761	**0.000****	**0.000****
MoCA	0.990	**0.000****	**0.000****
SDM	0.353	**0.000****	**0.000****
UHDRS TFC	0.773	**0.000****	**0.000****
PBA-s	0.633	**0.000****	**0.000****
HADS-SIS	0.241	**0.000****	**0.009****
TMS	0.261	**0.000****	**0.000****
TUG, seconds	0.424	**0.000****	**0.000****

Table S2. All Analysis of Covariance tests run while covarying for age, education, and weight.
Abbreviations: CRP = C-Reactive Protein; IL-6 = Interleukin 6; IL-1β = Interleukin 1βeta; *, p<0.05; **, p<0.01.

Table S2. ANCOVA Results in Salivary Inflammatory Biomarker Levels Among Diagnostic Groups (p-values)			
Inflammatory Marker	**NC vs PM**	**NC vs HD**	**PM vs HD**
CRP	**0.032***	0.337	0.226
Cortisol	0.346	0.179	**0.047***
IL-6	0.797	**0.024***	**0.048***
IL-1β	0.291	0.136	**0.011***
Amylase	0.305	0.238	0.910

Table S3. All partial correlations run while covarying for age, education, and weight. **Abbreviations**: CRP = C-Reactive Protein; IL-6 = Interleukin 6; IL-1β = Interleukin 1βeta; CAG = CAG repeat length; PAO = Parental Age of Onset; DBS = Disease Burden Score; MMSE = Mini Mental State Exam; MoCA = Montreal Cognitive Assessment; SDM = Symbol Digit Modalities Test; UHDRS TFC = Unified Huntington's Disease Rating Scale Total Functional Capacity; TMS = Total Motor Score; TUG = Timed Up and Go; PBA-s = Problem Behaviour Assessment-short form; HADS-SIS = Hospital Anxiety and Depression Scale – Snaith Irritability Scale; *, p<0.05; **, p<0.01.

Table S3. Partial Correlation between Demographic and Clinical Measures and Salivary Inflammatory Biomarker levels in Gene Positive Participants (n=87)					
Parameters	Cortisol	CRP	IL-6	IL-1β	Amylase
CAG					
r-value	0.390	-0.077	0.441	0.234	-0.105
p-value	0.030	0.529	0.004	0.117	0.349
PAO, yrs					
r-value	-0.002	0.144	-0.284	**-0.428**	0.023
p-value	0.992	0.275	0.143	**0.013***	0.853
DBS					
r-value	0.344	-0.063	**0.507**	**0.305**	-0.100
p-value	0.058	0.602	**0.001****	**0.039***	0.374
MMSE					
r-value	-0.152	-0.064	-0.101	-0.156	0.071
p-value	0.415	0.597	0.535	0.301	0.530
MoCA					
r-value	-0.162	-0.010	0.008	-0.231	-0.029
p-value	0.400	0.934	0.959	0.123	0.800
SDM					
r-value	-0.334	-0.034	-0.176	-0.201	0.040
p-value	0.066	0.780	0.277	0.181	0.720
UHDRS TFC					
r-value	-0.260	0.096	**-0.337**	**-0.358**	0.073
p-value	0.157	0.427	**0.033***	**0.014***	0.520
TMS					
r-value	0.251	-0.168	0.234	**0.436**	-0.045
p-value	0.173	0.164	0.146	**0.002****	0.690
TUG, sec					
r-value	0.026	-0.099	-0.085	-0.101	-0.173
p-value	0.899	0.454	0.631	0.533	0.147
PBA-s					
r-value	-0.100	-0.162	0.052	0.095	-0.106
p-value	0.591	0.181	0.748	0.528	0.347
HADS-SIS					
r-value	0.114	-0.045	0.082	0.127	-0.194
p-value	0.556	0.719	0.625	0.412	0.093