UNIVERSITY OF CALIFORNIA SAN DIEGO

Quantification and Analysis of Epigenetic Regulation of Brain-Derived Neurotrophic Factor (BDNF) in Huntington's Disease

A thesis submitted in partial satisfaction of the requirements
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in

Biology

by

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The Thesis of Ashley Michelle Gutierrez 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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ABSTRACT OF THESIS

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by

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Master of Science in Biology

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder that affects motor coordination and cognitive function. HD neuropathology is characterized by substantial loss of medium spiny neurons in the striatum. Brain derived neurotrophic factor (BDNF) plays fundamental roles in the survival and activity of neurons, including striatal cells that die in HD. Previous reports shown substantial alterations in BDNF levels in the brains of HD cases; however, limited studies have

explored the changes in BDNF across clinical stages of HD and levels present in peripheral fluids. We hypothesized that BDNF is increasingly deregulated during HD progression, and could be detected in plasma and saliva. Moreover, we propose that changes in *BDNF* gene expression may result from alterations in epigenetic mechanisms, specifically aberrant DNA methylation at the BDNF promoter; as previously reported to occur in other neurodegenerative disorders. We optimized an ELISA to measure BDNF in patients in different HD clinical stages and control subjects. We found lower levels of BDNF in saliva from subjects with the *HTT* gene mutation, regardless of HD clinical disease status in comparison to non-carriers. No changes in BDNF were found in plasma. We also determined the methylation levels at BDNF promoter IV by pyrosequencing. We found CpG sites differentially methylated in subjects with the *HTT* gene mutation. In summary, our preliminary studies suggest that BDNF protein levels and promoter methylation patterns may be associated with the huntingtin mutation and although they may be potentially indicative of the carrier status, fail to predict HD clinical progression.

1. Introduction

Huntington's disease (HD) is a neurodegenerative fatal genetic disorder that affects motor coordination and leads to cognitive decline. Nearly 3-7 in every 10,000 individuals are impacted by the autosomal dominant disorder (Ho et al., 2001). Symptoms usually arise at the age of 30 and are characterized by involuntary writhing movements known as "chorea" (Lanska, 2000). Individuals with the disorder become significantly impaired in both cognitive and physical abilities; consequently, leading to poor quality of life and inability to function in society. Presently, HD predictive testing is limited to a genetic test and has few peripheral biomarkers (Ross et al., 2014). As of now, there is no cure for the disease or adequate treatment.

HD is caused by a CAG trinucleotide repeat expansion in the first exon of the *HTT* gene encoding the HUNTINGTIN protein, located on Chromosome 4 (Macdonald et al., 1993). The abnormal increase in this trinucleotide repeat in the *huntingtin* gene leads to an expansion of over 35 glutamine repeats that leads to toxic aggregation of the HUNTINGTIN protein (Gil & Rego, 2008) . Length of the repeat correlates with HD age of death (Keum et al., 2016). The disease causes nerve cell death in the striatum and loss in neurogenesis (Ho et al., 2001). The accumulation of mutant HUNTINGTIN protein interferes with transcriptional regulators and other proteins expressed in the striatum and cortex. Mutant HUNTINGTIN interacts with proteins normally associated with wildtype HUNTINGTIN, such as calmodulin or huntingtin protein-associated protein, and generates toxic features of HD. Moreover, the mutant protein induces mitochondrial irregularities leading to further deregulation of transcription factors in HD (Sari, 2011). Overall, HD pathogenesis results in the loss of striatal and cortical integrity.

This loss of integrity led to the investigation of Brain-Derived Neurotrophic Factor (BDNF) in HD due to the protein's dependency on neurons in the striatum and cortex. BDNF production depends on striatal and cortical integrity as it is transported via cortical afferents to the striatum.

BDNF is a nine exon gene that results in multiple transcripts of the BDNF protein due to alternative splicing. The protein is associated with neuron proliferation and neurogenesis. An increase in BDNF results in an increase in neurogenesis, dendritogenesis, synaptogenesis, and synaptic strengthening. The protein binds to a tropomyosin-related kinase B (TrkB) receptor and p75 receptors. BDNF binding to TrkB results in dimerization and autophosphorylation initiating signal cascades, such as α-amino-3-NMDA receptor. These cascades aid in the promotion of neuronal growth and neuron plasticity (Binder & Scharfman, 2004). The neuronal growth and increase in neuron plasticity helps the brain adapt and learn. Low levels of BDNF results in synaptic failure, physical and cognitive deficits.

Researchers have found significant alterations in BDNF levels in individuals with neurological disorders (Steiner, Wolf, & Kempermann, 2006), and in association with the physical and neurological state of subjects. Physical activity and mental exercise elevate BDNF levels (Sleiman et al., 2016) while neurological diseases result in a deficiency in BDNF signaling (Autry & Monteggia, 2012). Studies show alterations of BDNF in progressive neurological disorders. BDNF levels vary based on transitional stages of Alzheimer's (O'Bryant et al., 2009) and Parkinson's disease (Costa et al., 2015). Lower levels of BDNF correlate with more advanced stages of the disease.

Studies indicate that loss of neurogenesis in neurological disorders may correlate with levels of BDNF suggesting additionally that one component that may be impacted by the neurodegenerative disorder of HD is Brain Derived Neurotrophic Factor (BDNF) (Steiner et al., 2006; Zuccato & Cattaneo, 2007). Studies detail lowered levels of BDNF in mice (Lynch et al., 2007; Zuccato et al., 2001) and human (Ferrer, Goutan, Marin, Rey, & Ribalta, 2000; Zuccato et al., 2001; Zuccato et al., 2008) models of HD. Investigation of decreased BDNF in HD models suggests that mutant HUNTINGTIN may hinder *BDNF* gene expression (Zuccato et al., 2001; Zuccato et al., 2003) or impair anterograde transit (Gauthier et al., 2004; Her & Goldstein, 2008). In postmortem HD brain samples, studies show a decreased amount of BDNF (Zuccato & Cattaneo, 2007). In transgenic mice models of HD, degradation of striatal neurons led to a reduction of BDNF protein that resulted in cognitive and physical impairments. On the other hand, over-expression of BDNF induced in the HD mice models resulted in phenotypical and cognitive rescue (Y. X. Xie, Hayden, & Xu, 2010). Mice injected with exogenous BDNF showed reduced weight loss, enhanced rotarod performance, and reversed striatal death. In addition, altered BDNF levels correlate with the length of the polyglutamine repeat in the huntingtin gene. However, limited studies on the relationship of BDNF levels with the stages of Huntington's Disease have been conducted so far. In a study in HD mice, a reduction of BDNF resulted in earlier onset of motor symptoms and exacerbated motor deficits (Canals et al., 2004). In the present study, we examined the levels of BDNF in plasma and saliva from subjects at different stages of HD, aimed at exploring the potential utility of BDNF as a peripheral biomarker for HD clinical manifestation.

In our study, we endeavored to quantify BDNF levels in human saliva and plasma in human control and HD patients. To our knowledge, there has been no successful quantification of BDNF in saliva of HD patients. Preliminary studies performed in our lab suggest that there is an association between lower BDNF concentration in saliva of individuals who are gene positive for HD. Our results examine the potential use of BDNF protein as a peripheral biomarker for HD clinical manifestation and confirm a relation between BDNF and HD progression, specifically in saliva.

The levels of circulating BDNF are ultimately regulated by a balance between transcription, translation and degradation (P. A. Desplats, 2015). In particular, epigenetic mechanisms like DNA methylation, that modulate chromatin structure, regulate gene expression (P. A. Desplats, 2015). Indeed, previous studies indicate the role of epigenetic modifications in alteration of BDNF transcription (Mitchelmore & Gede, 2014). Moreover, changes in DNA methylation at the BDNF promoter have been associated with reduced BDNF expression in neurological disorders such as Alzheimer's (B. Xie et al., 2017), major depression (Januar, Ancelin, Ritchie, Saffery, & Ryan, 2015) and schizophrenia (Ikegame et al., 2013). We therefore examined the role of epigenetic modifications on regulating BDNF levels in HD clinical manifestation. We investigated methylation levels on sites of the BDNF promoter associated with neurological disorders; specifically, sites in the promoter IV region (Favalli, Li, Belmonte-de-Abreu, Wong, & Daskalakis, 2012; Ikegame et al., 2013; Januar et al., 2015; Keller et al., 2010; B. Xie et al., 2017). The overall aim of our study was to investigate the potential role of BDNF as a peripheral biomarker for HD clinical

manifestation and the function of the methylation status of the BDNF promoter in altering BDNF levels.

2. Subjects and Methods

2.1 Regulatory

The study was approved by University of California, San Diego (UCSD) International Review Board in agreement with the requirements of the Code of Regulations on the Protection of Human Subjects. All participants gave informed consent prior to participation. Participants were recruited from University of California, San Diego (UCSD) HD Clinical Research Center.

2.2 Participants

This study was nested within a larger study of HD and control subjects participating in a study of Salivary Huntingtin Levels in HD patients at the UC San Diego Medical Center in San Diego. Three separate subject cohorts were used in this study to individually examine a) salivary BDNF n=43 b) plasma BDNF n=79 and c) BDNF methylation n=39. Participants were first stratified based on genetic testing for HD; specifically, for the polyglutamine expansion in the *huntingtin gene*. Control subjects, Non-Carriers (NC), consisted of individuals with no family history of HD who were negative for the HD gene mutation. NC participants had no history of psychiatric or neurodegenerative disorders nor did they use any psychoactive medications. Participants who tested positive for the HD gene mutation were stratified as HD Gene Positive (HD Gene +) group. We examined the following samples: a) saliva samples from patients HD Gene + (n=32) and NC (n=11) b) plasma samples from patients HD Gene + (n=51) and NC (n=28) and c) whole blood samples from patients HD Gene + (n=21) and controls (n=18). In the quantification of BDNF in the saliva and plasma cohorts, HD Gene + patients were further stratified based on their disease stage as

either Premanifiest HD (PM) or Manifest HD (HD). PM subjects were asymptomatic HD Gene + individuals. Manifest HD subjects were symptomatic HD Gene + individuals. In saliva, 18 PM and 14 HD samples were examined. In plasma, 30 PM and 21 HD samples were analyzed.

2.3 Sample Collection

For saliva sample collection, participants abstained from drinking, eating, smoking or use of oral hygiene products for at least one hour prior to collection. Thirty minutes before samples were collected, subjects thoroughly rinsed their mouth with water. Saliva samples were collected by the passive drool method between 9am and noon. Samples were then frozen immediately. In preparation for the assay, samples were thawed, centrifuged at 10,000 rpm for 10 minutes at 4°C and the supernatants were collected.

For plasma sample collection, blood was directly drawn into heparin-coated tubes and incubated at RT for 1 hour before centrifugation at 4500 RPM for 10 minutes to separate red blood cells. The top layer was collected, and 500ul aliquots were frozen immediately. Whole blood samples were immediately frozen after collection without previous fractionation.

2.4 BDNF Enzyme-Linked Immunosorbent Assay (ELISA)

BDNF levels in saliva and plasma was quantified by ELISA following a previously published method to produce in house assays (Mandel, Ozdener, & Utermohlen, 2011). Overall, 100ul of 1μg/ml solution of Anti-BDNF Mouse mAb was added to each well and incubated overnight at 4°C. After incubations, plates were washed eight times with Sample Buffer (1% TBS with 0.1% Tween), soaking 1 minute each wash. Next, 300μl of

Blocking Solution (3% BSA in TBS with 0.1% Tween) was added to each well and left at room temperature with agitation for 2.5 hours. After incubation, plates were washed 8X and 100μl of each sample was added to the appropriately wells in triplicates. Plates were then incubated for 2 hours at room temperature with agitation and then washed 8X. Next, 100μl of Chicken Anti-Human BDNF pAb was added to each well and left to incubate at room temperature for 2.5 hours with agitation. After incubation, plates were washed 8X and 100μl of anti-Chicken IgY-HRP was added to each and left to incubate for 1 hour at room temperature with agitation. Plates were washed again 8X and 100μl of TMB Solution was added to each well for 15 minutes. After, 100μl of 1M HCL was added to each well. Plates were read at 450nm and BDNF values were extrapolated from absorbance readings based on standard curve. Absorbance values from the 450nm reading were used in our analysis of salivary BDNF levels for 11 controls and 32 patients.

For determinations in saliva, samples were diluted 1:1 with Sample Buffer. Protease inhibitor (3μl) was added to each sample to avoid degradation of BDNF by salivary enzymes. Plasma samples were diluted 1:5 with Sample Buffer. BDNF concentration values (pg/ml) were extrapolated, using the standard curve, from the absorbance values from the 450nm reading.

2.5 Clinical Exams

Participants were assessed on the basis of motor, functional capacity, cognitive, and behavioral measures. Clinical exams were administered by certified raters and one neurologist (JCB) at UCSD Huntington's Disease Clinical Research Center.

2.5.1 Motor and Functional Capacity Measures

All participants were assessed with the Unified Huntington's Disease's Rating Scale (UHDRS), which is a standardized tool, consisting of multiple rating scales, used to assess cognitive, motor, behavioral and functional capacity in HD (Kieburtz et al., 1996). On the UHDRS Total Motor Score (TMS), the maximum score possible is 124 with higher scores indicating greater motor impairment. On the UHDRS Total Functional Capacity (TFC), which assesses the functional performance of participants, lower scores are indicative of poorer abilities.

2.5.2 Cognitive Measures

The MMSE tests five areas of cognitive function: orientation, registration, attention, calculation, recall, and language. There is a maximum possible total of 30 points and higher scores are indicative of better cognitive function.

2.5.3 Behavior and Emotional Distress Measures

All subjects were also assessed using two behavioral scales: the Problem Behaviors Assessment (PBA) and the Hospital Anxiety and Depression Scale (HADS), which recognize depression and further emotional disturbances. In both scales, a higher score is indicative of greater distress.

2.6 DNA Isolation

DNA was purified using QIAamp DNA Blood Midi Kit (Qiagen) as per manufacturer's protocol. DNA was extracted from 38 whole blood samples in total.

2.7 Pyrosequencing

Bisulfite Pyrosequencing was performed by EpigenDx Services. DNA was diluted to 20 ng/μl with water and 500 ng gDNA/sample were submitted for bisulfite conversion and pyrosequencing. Two probes, previously designed and validated by EpigenDx,

were used in the analysis of the BDNF promoter IV region. Probe ADS221-FS2re covered region chr11:27723246-27723191 including 6 CpG sites. In addition, probe Ads3858-FS mapped to chr11:27723144-27723076 and included 6 CpG sites. Methylation data was obtained for all 38 samples.

2.8 Data Analysis

The significance of the difference in salivary BDNF across diagnostic groups was examined by ANCOVA analysis, using age as a covariate. Mean levels of salivary BDNF in all diagnostic groups: NC, PM, HD were compared. A series of Spearman correlation tests were used to assess the relationship between salivary BDNF levels and clinical assessments, by comparing levels of BDNF (Absorbance) to raw scores in clinical measures. Data analysis was repeated with levels of BDNF (pg/ml) in plasma. Differences in age between diagnostic groups were controlled for in all analyses. A series of Student's t-test were used to assess the effect of HD gene mutation in methylation of sites on the BDNF promoter IV, by comparing the site mean methylation % in NC and HD Gene Positive subjects. Data represents mean value ± S.E.M.*$p<0.05$, unless otherwise stated.

3. Results

3.1 Subjects and Clinical Measures

Three different subject cohorts were used in this study to examine a) salivary BDNF in 43 subjects; b) plasma BDNF in 79 subjects and c) BDNF methylation in 39 subjects. The demographic and clinical data of the cohorts used for each BDNF analysis are described in **Tables 1-3**. Analysis of each individual cohort revealed a significant difference in age between groups of the salivary and plasma BDNF cohorts (ANOVA, $p = 0.0034$*)* No significant difference in age was found between diagnostic groups of the BDNF methylation cohort (ANOVA, $p = 0.737$).

Table 1: Demographic and Clinical Characteristics of Saliva Subject Cohort (N=43)

	NC	PM	HD	Overall p-value
n	11	18	14	
Gender, F:M	5:6	7:11	8:6	
Age, yrs	58.55 (40-77)	43.67 (19-66)	58.15 (38-76)	0.0034*
Weight, lbs	184.64 (106-306.4)	177.48 (119-283)	142.28 (94.8-202.8)	0.074
BMI	28.30 (17.9-45.9)	26.44 (20.2-42.4)	23.06 (17.9-30.7)	0.159
Education	14.70 (12-22)	15.78 (12-22)	12.46 (6-17)	0.020*

Table 2: Demographic and Clinical Characteristics of Plasma Subject Cohort (N=79)

	NC	PM	HD	Overall p-value
n	28	30	21	
Gender, F:M	14:14	17:13	13:8	
Age, yrs	57.14(37-76)	41.17 (19-66)	56.76 (36-75)	0.000**
Weight, lbs	171.16 (104-285)	167.46 (99.4-290)	151.4 (90-263)	0.267
BMI	27.41 (16.9-45.3)	23.41 (15.9-35.4)	25.98 (14.4-43.4)	0.088
Education	14.68 (12-22)	15.53 (6-22)	14.8 (5-24)	0.626

Table 3: Demographic and Clinical Characteristics of Methylation Subject Cohort (N=39)

	NC	HD Gene +	Overall p-value
n	18	21	
Gender, F:M	12:6	12:9	
Age, yrs	50.9 (26.0 - 79.0)	49.2 (30.0 - 78.0)	0.737

Mean scores for all clinical measures by diagnostic group are reported for the saliva and plasma subject cohorts in **Tables 4** and **5**, respectively. We examined raw scores from the MMSE, TFC, PBA, HADS, and TMS. In both plasma and saliva subject cohorts, mean MMSE, TFC, and TMS scores were significantly different in Manifest HD as compared to control (ANCOVA, $p < 0.05$) subjects. Mean MMSE, TFC, and TMS scores were also significantly different in PM as compared to Manifest HD (ANCOVA, $p < 0.05$) subjects. No difference was seen in any of the clinical exam scores between PM and NC in either the saliva or plasma subject cohorts (ANCOVA, $p < 0.05$).

Table 4: Clinical Measures for Saliva Subject Cohort, mean (range)

	NC	PM	HD	Overall p-value
MMSE	28.73 (27-30)	28.33 (24-30)	26.58 (23-30)	0.0246*
TFC	12.73 (12-13)	13 (13-13)	9.69 (7-13)	0.000**
TMS	3.3 (0-10)	2.94 (0-9)	34.17 (11-63)	0.000**
PBA	2.55 (0-11)	5.39 (0-33)	10.62 (0-36)	0.133**
HADS	13.09 (0-24)	18.18 (1-33)	19.67 (1-64)	0.500

Table 5: Clinical Characteristics for Plasma Subject Cohort, mean (range)

	NC	PM	HD	Overall p-value
MMSE	27.96 (25-30)	27.43 (20-30)	22.86 (20-30)	0.000**
TFC	12.93 (12-13)	12.57 (6-13)	8.24 (3-13)	0.000**
TMS	1.00 (1-7)	5.23 (0-64)	39.14 (14-70)	0.000**
PBA	5.41 (0-35)	9.65 (0-57)	14.38 (0-40)	0.190
HADS	13.81 (0-28)	19.58 (0-58)	(24.92 (0-62)	0.113

3.2 Salivary BDNF Levels Among Diagnostic Groups

We quantified BNDF levels in saliva using ELISA assays. Salivary BDNF levels for NC, PM, and HD were 0.95, 0.32, and 0.43, respectively (**Table 6).** Mean total BDNF levels in saliva of PM (ANCOVA, $p = 0.008$) and HD (ANCOVA, $p = 0.015$) were reduced compared to NC subjects (**Figure 1**). No significant difference was found between PM and HD groups (ANCOVA, $p = 0.717$). Since BDNF levels have been reported to differ between men and women, we examined whether the differences observed in BDNF across our groups were influenced by gender. We found no

significant differences in salivary BDNF levels between males and females in the NC (*p* = 0.514) or HD Gene + (p = 0.598) groups.

Table 6: Brain Derived Neurotrophic Factor (BDNF) in Saliva, mean (range)

	NC	PM	HD	Overall p-value
BDNF Absorbance	0.95 (0.09-2.63)	0.32 (0.01-0.581)	0.43 (0.06-0.95)	0.004**
n	11	18	14	

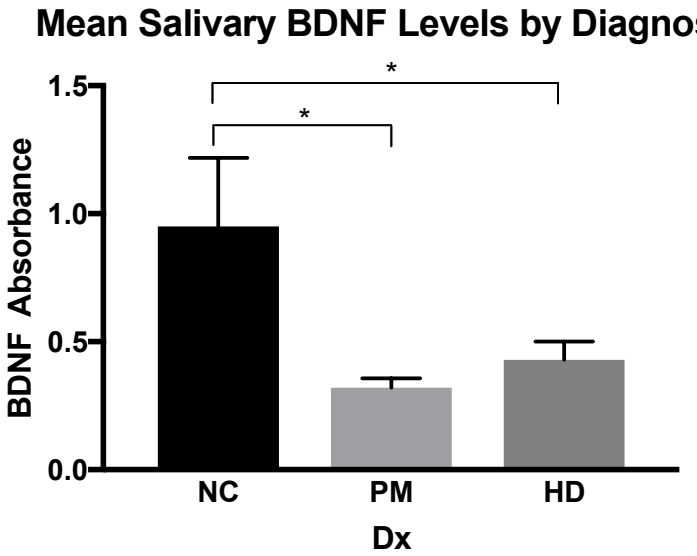


Figure 1: Distribution of BDNF levels in saliva by diagnosis group. BDNF levels were measured by ELISA. Data represents mean value ± S.E.M.*p<0.05 as per one-way ANOVA.

3.3 BDNF Levels in Plasma Among Diagnostic Groups

By means of ELISA, we successfully obtained concentration levels from plasma samples of 28 controls and 51 patients to analyze. Plasma BDNF levels for NC, PM, and HD were 235.48, 206.62, and 263.43, respectively (**Table 7)**. Mean total BDNF concentrations in plasma of PM (ANCOVA, *p* = 0.618) and HD (ANCOVA, *p* = 0.517) were not significantly different compared to NC subjects (**Figure 2**). No significant difference was found between PM and HD groups (ANCOVA, *p* > 0.05). Since BDNF

basal levels have been reported to differ between men and women, we again examined whether the differences observed in BDNF levels across our groups were influenced by gender. We compared plasma BDNF concentrations by gender and found no significant difference. We also found no significant differences in BDNF levels between males and females in NC (p= 0.843) and HD Gene + (p = 0.121) subjects.

Table 7: Brain Derived Neurotrophic Factor (BDNF) in Plasma, mean (range)

	NC	PM	HD	Overall p-value
BDNF (pg/ml)	235.48 (14.01-582.80)	206.62 (0.29-563.10)	263.43 (42.44-617.64)	0.004**
n	28	30	21	

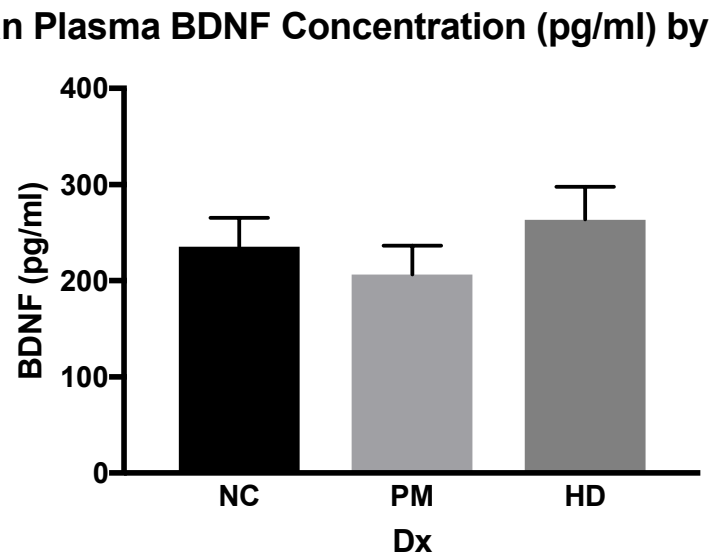


Figure 2: Distribution of BDNF levels in plasma by diagnosis group. BDNF levels were measured by ELISA. Data represents mean value ± S.E.M.*p<0.05 as per one-way ANOVA.

3.4 Relationship of BDNF levels in Saliva and Plasma with age and BMI

We used data from NC subjects to test possible correlations between BDNF levels and confounding factors. We observed significant correlations between plasma

BDNF concentration and age in our NC subjects (r = 0.506, *p = 0.006*), but no significant correlations between salivary BDNF levels and age (r = 0.345, *p = 0.298).* Since BDNF levels have been reported to differ based on body mass, we also examined whether the differences observed in BDNF across our groups were influenced by body mass. We found no significant correlations in either saliva (r = 0.406; *p* = 0.282) or plasma *(r = 0.272; p* = 0.179) in NC between BDNF levels and Body Mass Index (BMI).

3.5 Relationship of BDNF levels in Saliva and Plasma with Clinical Measures

Next, we looked for possible correlations between salivary and plasma BDNF levels and clinical measures. Correlations between clinical exams and BDNF levels in saliva and plasma are presented in **Tables 8** and **9**, respectively. There were no significant correlations between BDNF levels in either of the fluids and any of the clinical scores, including MMSE, TFC, TMS, PBA, and HADS.

Table 8: Correlations Between Saliva BDNF Levels and Demographic/Clinical Characteristics

	All Subjects (n=42)		**NC (n=11)**		**HD Gene + (n=31)**	
	Spearman's r	**p-value**	**Spearman's r**	**p-value**	**Spearman's r**	**p-value**
BDNF vs Age	0.071	0.655	0.345	0.298	-0.106	0.571
BDNF vs BMI	0.282	0.074	0.406	0.244	-0.189	0.308
BDNF vs MMSE	-0.074	0.644	-0.052	0.879	-0.071	0.708
BDNF vs TFC	-0.010	0.949	-0.258	0.443	-0.034	0.856
BDNF vs Total Motor	-0.013	0.935	0.169	0.640	0.060	0.751
BDNF vs PBA	0.233	0.138	0.410	0.210	0.213	0.250
BDNF vs HADS	0.038	0.817	-0.137	0.688	0.176	0.361

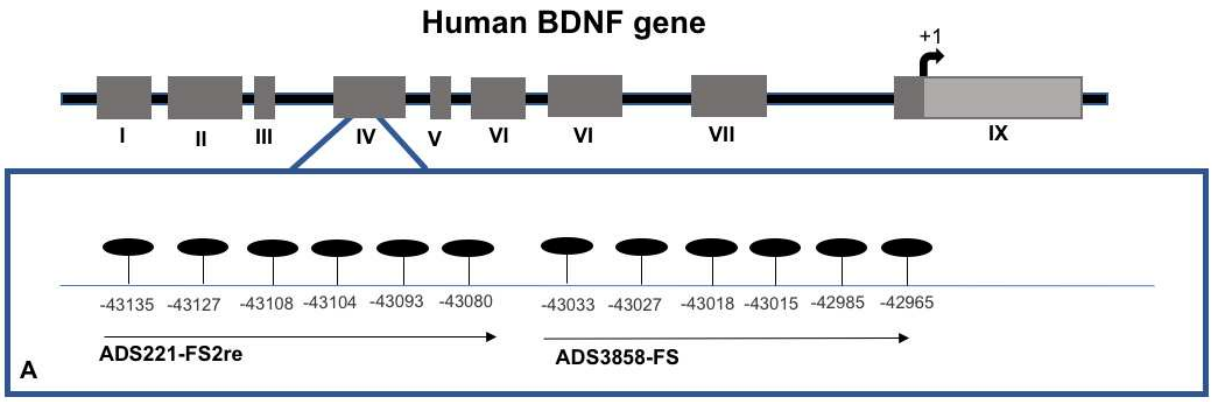
Table 9: Correlations Between Plasma BDNF Levels and Demographic/Clinical Characteristics

	All Subjects (n=79)		NC (n=28)		HD Gene + (n=51)	
	Spearman's r	**p-value**	**Spearman's r**	**p-value**	**Spearman's r**	**p-value**
BDNF vs Age	0.279	0.013*	0.506	0.006**	0.194	0.171
BDNF vs BMI	-0.087	0.453	0.272	0.179	-0.262	0.066
BDNF vs MMSE	-0.132	0.253	-0.321	0.102	-0.065	0.655
BDNF vs TFC	-0.055	0.634	0.089	0.657	-0.107	0.458
BDNF vs Total Motor	0.089	0.440	-0.117	0.56	0.152	0.292
BDNF vs PBA	0.114	0.407	-0.23	0.932	0.116	0.486
BDNF vs HADS	0.096	0.488	0.222	0.426	0.086	0.607

All subjects were cognitively and functionally assessed by a rater. BDNF levels were measured by ELISA. Data represents correlation coefficient value and p-values.*p<0.05 **p<0.01 as per Spearman partial correlation. *BMI= Body Mass Index; MMSE= Mini-Mental State Exam; TFC: Total Functional Capacity; PBA= Problem Behaviors Assessment; HADS-SIS=Hospital Anxiety and Depression Scale-Snaith Irritability Scale*

3.6 DNA Methylation levels at BDNF Promoter IV in Huntington's Disease Patients

We quantified methylation levels in whole blood samples by pyrosequencing. We examined methylation levels at the region of the BDNF Promoter IV, in DNA isolated from whole blood samples from 18 controls subjects and 21 HD Gene + patients**.** CpG sites and chromosomal locations are outlined in **Figure 3**. Mean methylation levels for each CpG site for NC and HD Gene Positive groups are reported in **Table 10.** Overall, the CpG analyzed present low methylation levels in both NC and HD subjects. We found 4 CpG sites differentially methylated in HD Gene + patients; three of the sites showed increased methylation (CpG 444-446) and one, CpG 437, showed decreased methylation levels (**Table 10**; **Figure 4**).



BDNF Promoter IV (chr11: 27701788-27701419)

27701788 CCTGGAACGGAACTCTTCTAATAAAAGATGTATCATTTTAAATGCGCTGAATTTTGATTC
27701728 TGGTAATTCGTGCACTAGAGTGTCTATTTCGAGGCAGCGGAGGTATCATATGACAGCGCA
ADS221-FS2re
27701668 CGTCAAGGCACCGTGGAGCCCTCTCGTGGACTCCCACCCACTTTCCCATTCACCGCGGAG
27701608 AGGGCTGCTCTCGCTGCCGCTCCCCCCGGCGAACTAGCATGAAATCTCCCTGCCTCTGCC
ADS3858-FS
27701548 GAGATCAAATGGAGCTTCTCGCTGATGGGGTGCGAGTATTACCTCCGCCATGCAATTTCC
27701488 ACTATCAATAATTTAACTTCTTTGCTGCAGAACAGAAGGAGTACATACCGGGCACCAAAG
B 27701428 ACTCGCGCCC

Figure 3: BDNF promoter IV region for methylation status. **(A)** CpG sites analyzed by each probe. Site position from ATG. **(B)** Regions of genome analyzed with probes were highlighted and underlined. A total of 12 analyzed CpG sites were labeled in red.

Table 10: DNA Methylation Analysis of BDNF Promoter IV Region

BDNF Region	Numbering	Chromosomal position	Methylation in NC [1]	Methylation in HD Gene +[1]	p-value[2]
Promoter IV	CpG #436	Chr11:27701529	1.2 (0.0 - 4.6)	1.3 (0.0 - 5.1)	0.890
	CpG #437	Chr11:27701549	4.1 (3.1 - 5.5)	2.9 (0.0 - 5.9)	0.032*
	CpG #438	Chr11:27701579	1.0 (0.0 - 4.2)	1.0 (0.0 - 4.1)	0.849
	CpG #439	Chr11:27701582	3.9 (0.0 - 5.7)	3.1 (0.0 - 7.7)	0.196
	CpG #440	Chr11:27701591	2.4 (0.0 - 4.4)	2.3 (0.0 - 5.8)	0.889
	CpG #441	Chr11:27701597	1.5 (0.0 - 3.8)	1.7 (0.0 - 4.2)	0.706
	CpG #444	Chr11:27701644	1.1 (0.0 - 3.5)	2.3 (0.0 - 7.0)	0.028*
	CpG #445	Chr11:27701657	0.9 (0.0 - 3.5)	2.1 (0.0 - 5.3)	0.023*
	CpG #446	Chr11:27701668	0.3 (0.0 - 2.6)	1.4 (0.0 - 3.7)	0.002**
	CpG #447	Chr11:27701672	2.9 (0.0 - 6.9)	2.9 (0.0 - 6.5)	0.961
	CpG #448	Chr11:27701691	5.3 (0.0 - 8.9)	6.4 (3.5 - 11.3)	0.079
	CpG #449	Chr11:27701699	3.2 (2.0 - 6.3)	3.7 (0.0 - 6.1)	0.312

1. Methylation levels are expressed as mean percentage and (min-max) values.
2. As per two-tailed Student's t-test in the comparison HD vs controls.

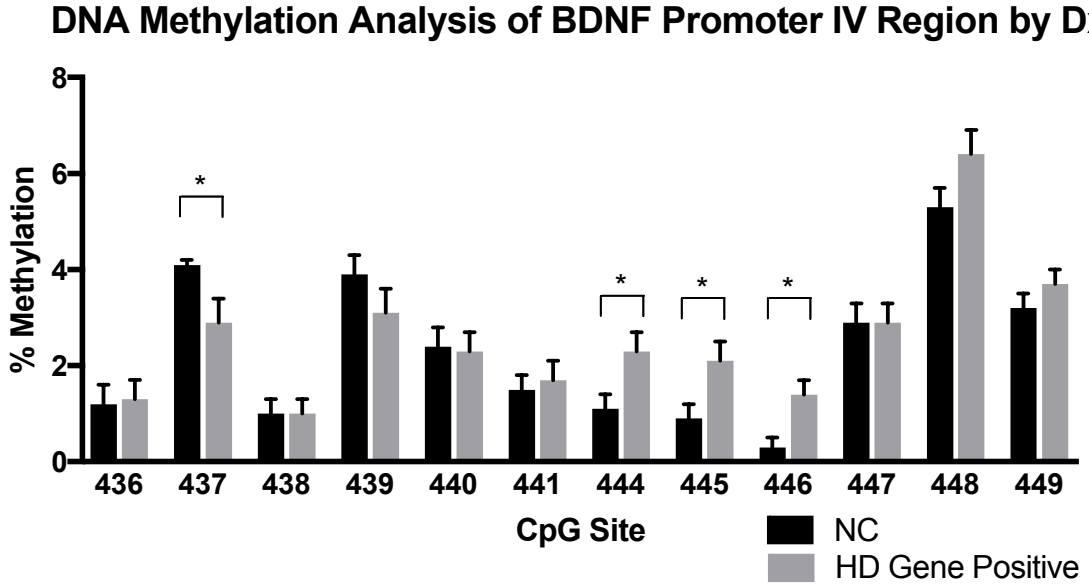
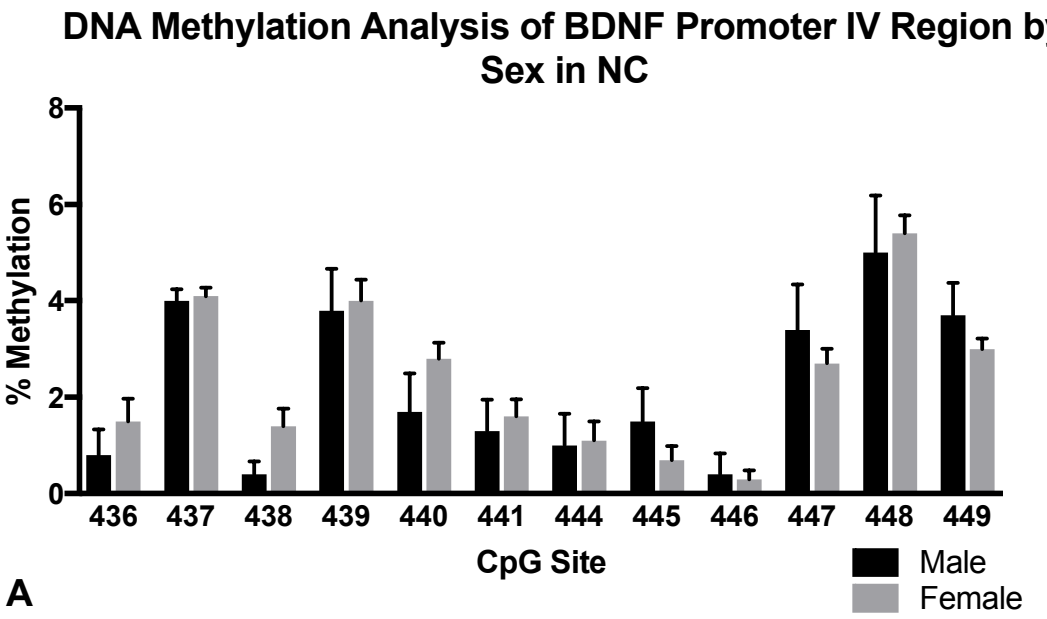


Figure 4: Distribution of methylation % on CpG sites by diagnosis group. Methylation levels were measured by pyrosequencing. Data represents mean value ± S.E.M.*p<0.05 as per two-tailed Student's t-test.

3.7 Relationship of DNA Methylation levels at BDNF Promoter IV with age and gender

We used BDNF methylation values to look for possible correlations with age and gender. We found no significant correlation between age and methylation levels ($p > 0.05$). Analysis of BDNF methylation levels by sex in NC and HD Gene + groups, revealed no significant differences ($p > 0.05$) between females and males (**Figure 5**).



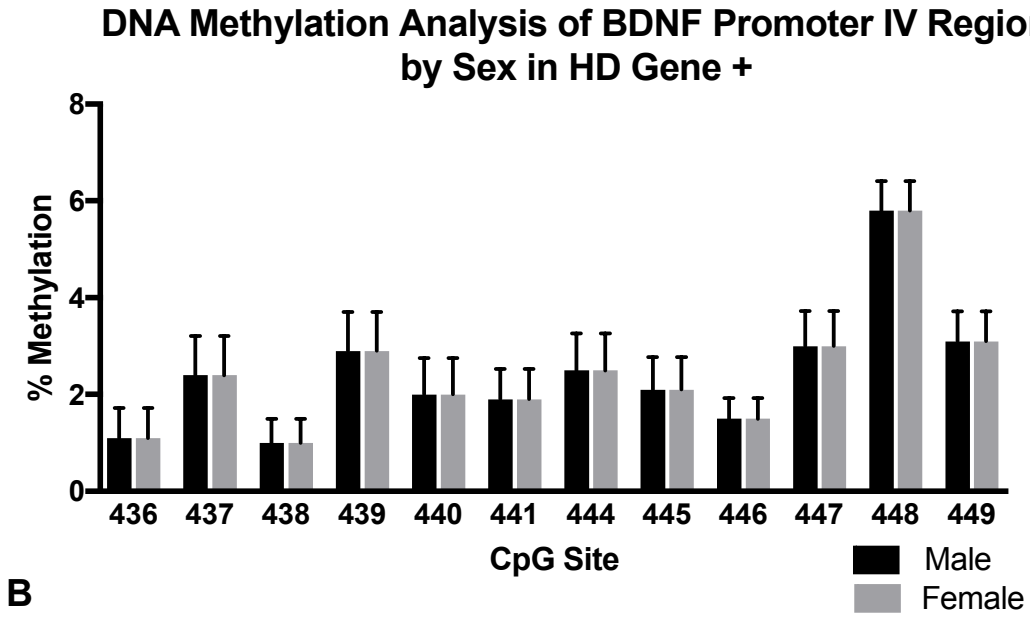


Figure 5: Distribution of methylation % on CpG sites by sex in NC **(A)** and HD Gene Positive **(B)**. Methylation levels were measured by pyrosequencing. Data represents mean value ± S.E.M.*p<0.05 as per two-tailed Student's t-test.

4. Discussion

This study examines the relationship between BDNF levels and the clinical manifestation of HD in two ways: 1) quantification of the actual protein in peripheral fluids and 2) investigation of the epigenetic regulation of the gene encoding BDNF. To our knowledge, our study is the first to quantify BDNF in saliva and the first to study methylation of BDNF in patients with the *huntingtin* mutation. Results of this study replicated previously reported BDNF levels in plasma in HD patients. Additionally, our study explores the potential use of BDNF as a biomarker of the clinical manifestation of HD. We examined the potential of BDNF to predict the clinical status of subjects with, and at risk for, HD. Our findings suggest that BDNF may have a role as a biomarker in HD.

Previous studies have suggested the potential use of saliva as a diagnostic tool (Kaufman & Lamster, 2002). With respect to BDNF in saliva, we believe our study is the first to relatively quantify the protein in patients with HD. In this study, we analyzed a total of 11 NC, 18 PM HD and 14 Manifest HD subjects. Further stratification of HD Gene + groups and exploration of potential differences between manifest HD and PM subjects, is significant with regard to clinical care. Cognitive and motor performance, using clinical exams, such as the MMSE and UHDRS TMS, of individuals with the *huntingtin* mutation were evaluated to stratify patients as asymptomatic (PM) and symptomatic (Manifest HD). Differences between the two groups may serve as a valuable tool for predicting when patients may be close to conversion from asymptomatic to symptomatic. We detected relatively lower levels of BDNF in the saliva of patients with the *huntingtin* mutation but failed to find differences in salivary BDNF

levels between asymptomatic and symptomatic patients. Our findings suggest that BDNF levels may be altered by the *huntingtin* mutation, but may not have any relevancy with regard to symptom onset. However, since our saliva assay had low sensitivity and did not reach the levels of the standards, it may suggest that BDNF may still have a role in disease transition and that further investigation is needed. Our results support the previous report of altered BDNF levels in HD brains due to loss of BDNF synthesis in the striatum and cortex. Mutated HUNTINGTIN has been shown to alter BDNF gene transcription and protein levels as a result of damage to the striatal-cortical pathway, the main source of BDNF, in HD transgenic mice models (Zuccato et al., 2001). The same study performed by Zuccato et al. also observed decreased BDNF production, specifically in the striatum and cortex, in brain samples from human patients with HD. Mutant HUNTIGTIN binds to proteins, such as $p150^{glued}$ by HAP1, and inhibits proper transport of BDNF by vesicles in the corticostriatal axes (Baydyuk & Xu, 2014). Thus, a larger sample size may be necessary to make any conclusion with regard to salivary BDNF levels in patients with HD.

With regard to levels of BDNF in plasma, BDNF concentration did not vary between the diagnostic groups of NC, PM, and HD. Our study agrees with previous findings of changes of BDNF in plasma samples from patients with HD. A previous study suggested that plasma BDNF was not indicative of HD diagnosis (Zuccato et al., 2011). The study utilized a commercially available kit and relatively quantified plasma BDNF levels. In the current study, we successfully used a previously reported (Mandel, Ozdener, & Utermohlen, 2011) in-house ELISA system to quantify BDNF levels in the plasma of 28 NC, 30 PM HD and 21 Manifest HD subjects. We found no differences in

plasma BDNF between diagnostic groups. One study suggested that BDNF levels in plasma may differ between females and males, with women having significantly higher levels (Lommatzsch et al., 2005); however, we did not find significant gender differences in plasma BDNF in our cohort, regardless of disease status. Our analysis is limited due to uneven gender distribution in each diagnostic category and further investigation is needed. Our findings suggest that BDNF levels in plasma are not reflective of an individual's mutation status. This may suggest that BDNF changes associated with the HD mutation cannot be measured in plasma, perhaps due to different transcriptional regulation in plasma compared to brain. BDNF is a complex unit intricately regulated and formed of nine different promoters (Pruunsild, Kazantseva, Aid, Palm, & Timmusk, 2007). Different isoforms of BDNF are found in each fluid. Zuccato et al. reported lowered transcription by promoters II, IV, VI in the HD brain (Zuccato & Cattaneo, 2009). However, BDNF transcripts detected in plasma are only of isoform IX (Zuccato et al., 2011). Therefore, BDNF changes seen in the HD brain, from striatal and cortical damage, may be absent from the blood due to different transcriptional regulations in each fluid. A larger sample size is needed before making conclusions regarding BDNF in plasma of patients with HD.

It is not known why BDNF levels of patients with the *huntingtin* mutation differs from NC in saliva but not in plasma. Unfortunately, in our study the plasma and saliva cohorts featured distinctly separate subjects and did not overlap. Therefore, we could not analyze the relationship between salivary and plasma BDNF for a given subject. To this aim, future studies should be performed using matching samples from the same subjects. Further investigation would indicate if perhaps salivary BDNF reflects systemic

processes, as seen in blood, and if BDNF holds the same promise as other salivary biomarkers (Williamson, Munro, Pickler, Grap, & Elswick, 2012).

BDNF expression may vary as a result of circadian regulation (Bova, Micheli, Qualadrucci, & Zucconi, 1998). In a study by Piccinni et al., significantly lower levels of plasma BDNF were seen in males at 22:00h of the day. However, in the same study, no daily variations were seen in plasma samples of females (Piccinni et al., 2008). In another study of plasma BDNF and circadian rhythms, plasma BDNF levels in males were found to be influenced by clock time while no influence on female BDNF levels were reported (Cain et al., 2017). Therefore, variations in time of sample collection may impact measured BDNF values and in further investigations samples should be collected at consistent times during the day. Furthermore, variation in sample collection day may influence BDNF levels. Research suggests there may be changes in BDNF levels based on the season in which samples are collected. In serum, BDNF levels in samples collected during the summer season were elevated compared with those collected during fall-winter (Molendijk et al., 2012). Lastly, length of storage of our samples varied and may have influenced the measured BDNF values. Altered storage length of samples has been found to affect quantified BDNF values (Zuccato et al., 2011).

Research suggests a possible association between BDNF level and age. Studies show a decrease in BDNF levels with increased age (Erickson et al., 2010; Lommatzsch et al., 2005); however, in our study, analysis of plasma BDNF and age revealed a moderate positive relationship. We further evaluated the potential relationship between BDNF levels and clinical assessment scores. We found no correlations between BDNF

levels with any clinical exam measurements, which questions its use as a marker of HD disease progression.

Multiple studies have assessed the role of epigenetic alterations in neurodegenerative disorders (Cronin et al., 2017; P. Desplats et al., 2011; Landgrave-Gomez, Mercado-Gomez, & Guevara-Guzman, 2015). In humans, *BDNF* is composed of nine promoters that contribute to tissue specific transcriptional regulation. Methylation of DNA is associated with reduction of gene transcription and expression (Razin & Cedar, 1991). Methylation of the promoter regions blocks the binding of transcription factors; ultimately, inhibiting transcription. Elevated methylation of BDNF promoters results in reduction of transcription of *BDNF* and protein levels (Lubin, Roth, & Sweatt, 2008). Identification of methylation patterns has been used as a diagnostic tool and as an accurate biomarker for cancers, neurodegenerative and psychiatric disorders (Levenson, 2010). Increased methylation at BDNF promoter I was associated with lower levels of BDNF in peripheral blood of schizophrenia patients in comparison to control subjects (Ikegame et al., 2013). Similarly, increased methylation at BDNF promoter was associated with lower levels of BDNF in post-mortem brains from Alzheimer's disease cases (Rao, Keleshian, Klein, & Rapoport, 2012). Higher methylation of the BDNF promoter, on exon I, has also been found to be associated with the clinical manifestation of AD, as characterized by negative correlations between BDNF levels and neuropsychological scores (Nagata et al., 2015). In a study of BDNF promoter IV, higher methylation was also found in patients with AD (B. Xie et al., 2017). Although there have been studies of the same methylation sites on the BDNF Promoter IV, the pattern of methylation at these sites in patients with the *huntingtin* mutation (HD Gene

+) have not been explored, and to the best of our knowledge, our study is the first to quantify the methylation in patients with HD. In this study, we observed significantly higher DNA methylation at CpG sites 444-446 on the BDNF promoter IV. Importantly, the three sites showing altered methylation are in close genomic proximity, suggesting these methylation sites may represent a functional unit that modulates BDNF transcription (Lovkvist, Dodd, Sneppen, & Haerter, 2016). In our study, we quantified BDNF methylation from DNA isolated from whole blood samples, therefore containing multiple lymphocyte types. Since methylation profiles are cell-specific, these heterogeneous samples may reduce signal to noise ratios and further studies could benefit from isolation of major cell types before methylation analysis. Nonetheless, the mean methylation values we observed in our study for control cases are in good agreement with methylation values reported for the same CpG sites in a previous study by Ikegame et al. investigating changes in BDNF in peripheral blood (Ikegame et al., 2013). Future studies are needed to investigate the role of elevated methylation on CpG sites on BDNF expression.

Sex and age are two factors known to affect DNA methylation in BDNF (Chan & Ye, 2017; Ihara et al., 2018). A recent study by Ihara et al. suggests significant correlations between DNA methylation and age. In this study, we did not find a significant correlation between DNA methylation and age, regardless of diagnosis. A study by Chan & Ye reported an increase in BDNF DNA methylation in females due to the presence of estrogen. In this study, we did not observe a difference in DNA methylation on Promoter IV between females and males, regardless of diagnosis. Although preliminary, our results suggest that there may be increased methylation on

several sites at the BDNF Promoter IV in patients with the *huntingtin* mutation, regardless of sex and age.

The interpretation of our results is limited by the size of our cohort, the uneven distribution of gender in each cohort, and the lack of overlap between cohorts. Further studies to validate our findings will need a single cohort with a larger number of subjects to study BDNF in saliva, in plasma, and methylation. Furthermore, results of the salivary assay were frequently below detection levels. Attempts to resolve the issue through an increase in washes, changes in dilutions and new reagents were unsuccessful.

In summary, our preliminary studies suggest that BDNF protein levels and promoter methylation patterns may be associated with the *huntingtin* mutation and although they may be potentially indicative of the carrier status, fail to predict HD clinical progression.

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