Chapter 12
Salivary Biomarkers and Neurodegenerative Conditions

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Abstract A major drawback for biomarker research on neurodegenerative diseases is that the pathologically affected tissue, i.e., the brain, cannot easily be accessed for sampling or biopsy. Hence, researchers have turned to peripheral tissues as biospecimens for such studies. The most commonly used biofluid is blood (serum or plasma), although urine and sweat have also been studied. This chapter will focus on the use of saliva as a biofluid for biomarker research studies on neurodegenerative diseases. Saliva is known to contain an abundance of hormones, proteins, and nucleic acid components that reflect physiological function, including several neurodegenerative disease-related proteins such as tau, amyloid beta, alpha-synuclein, and the huntingtin protein. Levels of these proteins in saliva have been proposed to represent useful biomarkers for their indicated diseases. This chapter will review studies demonstrating the presence of central nervous system proteins in saliva and the potential for salivary proteins to serve as biomarkers for neurological and neurodegenerative disorders, with a focus on three of the most common neurodegenerative diseases: Alzheimer's disease, Parkinson's disease, and Huntington's disease.

Keywords Central nervous system · Biomarker · Neurodegenerative disease

12.1 Background

Neurodegenerative diseases represent a broad family of diseases afflicting both the central and peripheral nervous systems. A primary feature of all neurodegenerative diseases is a progressive loss of certain classes of neurons that affect either motor function or memory and cognition. The degeneration of these neurons can be due to several molecular mechanisms that promote cell death including excitotoxicity,

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mitochondrial dysfunction, and intracellular inclusions or extracellular aggregation of toxic molecules (Dong, Wang, & Qin, 2009; Ross & Poirier, 2004; Trushina & McMurray, 2007). For genetic neurodegenerative diseases, such as triplet repeat disorders, diagnoses can be confirmed by genetic testing. However, for most other neurodegenerative diseases, diagnosis can be challenging and is usually based on symptom presentation. In either case, however, early detection is crucial for improved prognosis and optimal therapeutic outcomes.

There is a great need to identify biomarkers for these disorders for several reasons, including determining disease risk and onset, assessing the severity of symptoms, predicting disease outcomes and to track therapeutics. However, because sampling of central nervous system tissue is not possible, researchers have increasingly focused on other sources for biomarker studies. Cerebral spinal fluid (CSF) is thought to represent the biofluid most like the brain environment, however, CSF collection is a highly invasive technique that requires a lumbar puncture, which can be painful and lead to side effects and complications (Evans, 1998) (Table 12.1).

Table 12.1 Different types of biofluids used for biomarker research in neurodegenerative conditions

Biomarker type	Advantages	Disadvantages
CSF-based biomarkers	Reflects disease and pathological changes in the brain	Requires invasive lumbar puncture
	Does not require special laboratory equipment for analysis	Not suitable for all patients (i.e., elderly and those with advanced disease)
		Variability of analytical procedures across laboratories
		Normal biomarker range difficult to establish
Blood-based biomarkers	Easily available for analysis	Requires trained personnel to obtain sample
	Minimally invasive	Still invasive
	Cost-effective assays involved	Large starting volumes typically required
	Multiple assessments possible	Normal biomarker range difficult to establish
	Broad applications for diagnostics and monitoring therapeutic outcomes	Variability of analytical procedures across diagnostic laboratories
Saliva-based biomarkers	Noninvasive	Disease analytes present at low levels
	Does not require specially trained personnel	Possible matrix effects
	Can collect samples in any setting; minimal sample processing	Variability of analytical procedures across diagnostic laboratories
	Broad applications for diagnostics and monitoring therapeutic outcomes	

The advantages and disadvantages of each biofluid are summarized
CSF cerebrospinal fluid

With regards to peripheral sources, investigations in blood have dominated the field for decades; however, blood sampling is also an invasive technique that has several drawbacks (Table 12.1), notably the need for large starting volumes and the need for trained personnel to collect the blood samples. These drawbacks necessitate the pursuit for more advanced and less invasive testing options and have opened the doors for other biofluids, such as saliva, to serve as a source for biomarker analyses.

Like blood, saliva contains a multitude of constituents, including hormones, proteins, and nucleic acids that reflect biological functions (Yan et al., 2009). Unlike blood, which is typically similar in composition throughout the body, saliva is a composite of oral fluids secreted from many different sources with most originating from the major salivary glands, which include the parotid, submandibular, and sublingual glands (Baum, 1993; Granger et al., 2012) (Fig. 12.1). The minor salivary glands, which comprise ~600–1000 glands distributed throughout the oral cavity, are also important components of saliva secretion (Baum, 1993; Granger et al., 2012) (Fig. 12.1).

In general, human salivary glands produce ~750 ml of serous and mucinous saliva daily. This output, which is considered "whole saliva," consists of water, salts, and an abundance of molecules from the blood, as well as salivary proteins in the oral cavity (Humphrey & Williamson, 2001). Saliva secretion is influenced by many factors, which include the diurnal cycle, autonomic nervous system activity, exercise, and chewing. Medications, other treatments, and various medical conditions can also affect saliva secretion (Granger et al., 2012).

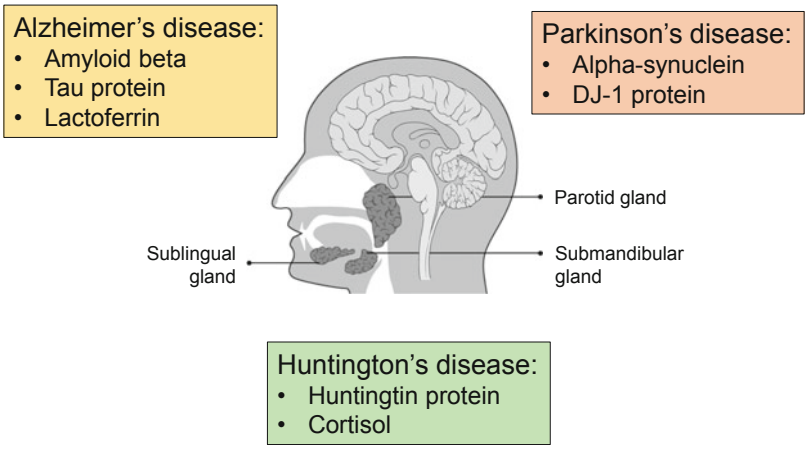

Fig. 12.1 Summary of the most promising salivary biomarkers for Alzheimer's disease, Parkinson's disease, and Huntington's disease. Biomarkers for each disease are listed. Saliva secretion occurs in the mouth by the actions of the major salivary glands, the parotid gland, submandibular gland, and sublingual gland, which are shown in the cartoon. The minor salivary glands (not shown) also contribute to saliva content and are most concentrated along the buccal, labial, and lingual mucosa, as well as in the soft and hard palates of the mouth

The use of saliva as a biofluid has several advantages over blood sampling (Kaczor-Urbanowicz et al., 2017; Pfaffe, Cooper-White, Beyerlein, Kostner, & Punyadeera, 2011). Most importantly, whole saliva is easy to collect in a noninvasive way. Further, compared to blood sampling, whole saliva collection requires no specially trained personnel, and is easier to process because it does not coagulate like blood. Further, providing a saliva sample reduces discomfort and anxiety for the patient and simplifies serial sample collection over long periods of time, such as in a clinical trial or testing at home (Table 12.1). Saliva collection can be considered safer than blood collection with regards to the risk for hepatitis and HIV (Campo et al., 2006; Wormwood et al., 2015), because needles are not used.

As a diagnostic fluid, saliva has been assessed in a growing number of studies for several pathological conditions, such as celiac disease (Lenander-Lumikari, Ihalin, & Lahteenoja, 2000), rheumatoid arthritis (Helenius et al., 2005), HIV (Holmstrom, Syrjanen, Laine, Valle, & Suni, 1990; Matsuda, Oka, Honda, Takebe, & Takemori, 1993), diabetes mellitus (Belazi, Galli-Tsinopoulou, Drakoulakos, Fleva, & Papanayiotou, 1998; Lopez et al., 2003), breast cancer (Streckfus & Bigler, 2005), Sjögren's syndrome (Ryu, Atkinson, Hoehn, Illei, & Hart, 2006), as well as for therapeutic drug monitoring (Drobitch & Svensson, 1992) (see Chap. 17). However, the use of saliva for biomarker studies on CNS disorders is a relatively new field, despite a report dating back to 1980 suggesting that salivary levels may reflect changes in CSF (Scherber, Richter, & Schaps, 1980). Later studies demonstrating the expression of CNS disease-related proteins in salivary epithelial cells and salivary glands helped promote the use of saliva for biomarker research for these disorders (Oh & Turner, 2006; Sousa, do Amaral, Guimaraes, & Saraiva, 2005). With the characterization of the salivary proteome, which was found to contain more than 2000 proteins and peptides (Hu, Loo, & Wong, 2007), the number and utility of saliva analytes for medicine are expected to expand substantially.

This chapter will summarize studies that have used saliva for biomarker research in neurodegenerative diseases, with a specific focus on Alzheimer's disease (AD), Parkinson's disease (PD), and Huntington's disease (HD) (Fig. 12.1). Although saliva bioscience research as it relates to brain conditions is a field in its infancy, the goal of this chapter is to provide a summary, to date, of the advances made in this area of research in hopes of promoting the use of saliva for nervous system research.

12.2 Saliva Studies in Neurodegenerative Conditions

12.2.1 Alzheimer's Disease: Clinical Characteristics and Pathology

Alzheimer's disease (AD) is the most common neurodegenerative disorder that currently afflicts approximately 2% of the general population, with the risk substantially increasing in individuals 70 years or older (Scheltens et al., 2016). Because life

expectancies are increasing in human populations, it is estimated that the number of individuals afflicted with AD will double in the next 10–15 years, becoming one of the leading causes of disability and death among the elderly (Alzheimer's Association, 2010; Ferri et al., 2005), not to mention the enormous economical cost to society. AD is characterized by the gradual loss of memory, inability to learn new things, deficits in speech and language, and in recognition of people and objects. Additionally, depression, delusions, and other psychiatric conditions can be present. AD is progressive in nature and eventually leads to a complete incapacity and death of the patient within 8–10 years of diagnosis (Magalingam, Radhakrishnan, Ping, & Haleagrahara, 2018). Major degeneration of neurons in AD occurs particularly in the basal forebrain, hippocampus, and other cortical areas of the brain, and dysfunction in these areas accounts for a majority of the symptoms associated with this disease.

AD can be classified into familial and sporadic forms. Familial AD comprises about 5% of AD cases and is due to mutations in the Amyloid beta precursor protein (*APP*) gene, Presenilin-1 (*PSEN1*), and Presenilin-2 genes (*PSEN2*) genes (Lanoiselee et al., 2017). The gene products of *PSEN1/PSEN2*, the presenilins, are components of the gamma-secretase multicomplex, which is responsible for the cleavage of the APP protein and the formation of amyloid beta peptides. Sporadic forms of AD show no family history, but some cases may be associated with the expression of apolipoprotein E4, a protein involved in the transport of lipids, cholesterol, and other hydrophobic molecules into the brain (Raber, Huang, & Ashford, 2004). In addition, brain changes associated with depressive episodes that compromise the ability of the brain to cope with stress may constitute risk factors for the development of AD (Aznar & Knudsen, 2011). Despite enormous progress in AD research over the past few decades, the precise cause of AD is not completely understood, and currently, there is no effective treatment for the disease.

12.2.2 AD Disease-Related Proteins: Amyloid Beta and Tau

Two main pathological hallmarks of AD are extracellular plaques, consisting of insoluble aggregates of the amyloid beta protein, and intracellular neurofibrillary tangles, composed of hyperphosphorylated tau protein (Magalingam et al., 2018; Scheltens et al., 2016). Although the exact nature of how these plaques and tangles are formed is not entirely clear, one well-established and detrimental consequence is neuronal loss and synaptic dysfunction in the AD brain. The normal function of the APP protein is not clear but it is believed to play important roles in memory formation, lipid homeostasis, and the regulation of neuronal activity and neurite outgrowth (Mitsuyama et al., 2009). Amyloid beta pathology arises from the abnormal cleavage of the APP protein, resulting in amyloid beta monomers and oligomers that aggregate ultimately forming amyloid beta fibrils and plaques (Lanoiselee et al., 2017). Dysregulated amyloid beta homeostasis is thought to represent an early event in AD pathology, as studies have shown that plaques can accumulate for up to 10 years before the onset of AD symptoms (Masters et al., 1985). Another argument

in favor of a role for amyloid beta in AD pathology is that both familial and sporadic forms of AD are associated with increased levels of this peptide (Dorszewska, Prendecki, Oczkowska, Dezor, & Kozubski, 2016).

The second core pathology is the formation of neurofibrillary tangles, which result from the hyperphosphorylation of tau, a microtubule-associated protein that functions in the cytoskeletal network and maintains proper neuronal structure and intracellular transport (Richter-Landsberg, 2016; Scholz & Mandelkow, 2014). In AD, phosphorylation of tau at multiple sites results in its removal from the microtubule causing disruption of microtubule structures, which leads to dysregulation of a number of neuronal processes ranging from protein trafficking to cellular morphology (Ulrich et al., 2018; Weingarten, Lockwood, Hwo, & Kirschner, 1975). The aggregation and toxic deposition of tau, and the ensuing formation of the characteristic neurofibrillary tangles, leads to compromised cellular function and ultimately, neuronal death (Braak & Braak, 1991; Maeda et al., 2007).

To date, CSF measures of amyloid beta, total tau, and phosphorylated tau proteins represent the most promising biomarkers for AD. Specifically, decreased levels of beta amyloid and increased levels of tau and phosphorylated tau in the CSF are the most reproducible biomarkers for AD diagnosis (Jack et al., 2018; Scheltens et al., 2016) (Table 12.2). Previous studies have successfully measured these in blood samples from AD patients, showing results generally consistent with the CSF findings (Jack et al., 2018; Scheltens et al., 2016) (Table 12.2). In addition to measurements of these disease-related proteins, biomarkers identifying complex

Table 12.2 Comparison of neurodegenerative biomarker measures in CSF, blood, and saliva

Disease	Biomarker	Effect in CSF	Effect in plasma/serum	Effect in saliva
Alzheimer's disease				
	Tau-total	↑	↑	↔
	Tau-phospho	↑	↑	↑
	Aβ42	↓	↔	↑ or ↔
	Aβ40	↔	↔	↔
Huntingon's disease				
	Htt-total	Not tested	↓ or ↔	↑
	Htt-mutant	↑	↑	Not tested
Parkinson's disease				
	Alpha-syn-total	↓	↔	↓ or ↔
	Alpha-syn-oligo	↑	↑	↑
	DJ-1	↓	↔	↑ or ↔

Up and down arrows depict the overall change in levels of the biomarker in patients with the disease compared to normal controls, based on previous studies. ↔, not significantly different between patients and controls. Data summarized from Devic et al. (2011), Lin et al. (2012), Weiss et al. (2012), Massai et al. (2013), Al-Nimer et al. (2014), Masters et al. (2015), Southwell et al. (2015), Wild et al. (2015), Vivacqua et al. (2016), Kang et al. (2016), Hijioka et al. (2017), Pchelina et al. (2017), Tatebe et al. (2017), Goldman et al. (2018), An et al. (2018), Corey-Bloom et al. (2018) and from https://www.alzforum.org/alzbiomarker

Htt huntingtin, *Alpha-syn* alpha-synuclein, *Alpha-syn-oligo* alpha-synuclein oligomeric, *Aβ* amyloid beta

pathways contributing to pathology in AD would be highly useful, especially for early AD patients. Because of the aforementioned advantages of saliva as a biospecimen, several studies have measured salivary levels of amyloid beta and tau in AD patients. These will be discussed below. Further, lactoferrin is another important inflammation-related protein that has been measured in saliva, which is also discussed below. Such noninvasive biomarkers could also have therapeutic applications, given that several AD therapeutic approaches have targeted the amyloid beta peptide and tau pathways (Barten & Albright, 2008).

12.2.3 Salivary Biomarkers in AD

12.2.3.1 Salivary Amyloid Beta Studies

The amyloid beta protein is typically a 40 amino acid long peptide, but lengths can range from 38 to 43 amino acids, with different forms being measured in different studies. The first study to measure amyloid beta in saliva used an enzyme-linked immunosorbent assay (ELISA) to compare amyloid beta 40 (Aβ40) and amyloid beta 42 (Aβ42) levels between AD patients and two groups of controls: normal healthy subjects and Parkinson's disease patients (Bermejo-Pareja, Antequera, Vargas, Molina, & Carro, 2010). Results showed a small, but statistically significant, increase in salivary Aβ42 levels in mild and moderate AD patients compared to both of the control groups, but no differences in saliva concentrations of Aβ42 in patients with severe AD compared to the control groups (Bermejo-Pareja et al., 2010). In contrast, no changes in salivary levels of Aβ40 were detected among the three cohorts of subjects. Further, this study showed that the association between saliva Aβ42 levels and AD was dependent on gender and associated with Total Functional Capacity clinical scores, but independent of known risk factors, such as age or the *APOE4* genotype (Bermejo-Pareja et al., 2010). In another study using an antibody-based magnetic nanoparticle immunoassay, in addition to an ELISA method, increases in both Aβ40 and Aβ42 were detected in AD patients (Kim, Choi, Song, & Song, 2014). However, there was no difference in Aβ42 concentrations with disease progression, ranging from mild cognitive impairment to severe AD symptomatology (Kim et al., 2014), an effect also observed by Bermejo Pareja and colleagues (Bermejo-Pareja et al., 2010). The fact that PD patients showed no significant difference in Aβ42 levels compared to healthy controls suggests specificity for salivary Aβ42 to serve as a biomarker for AD patients.

Another recent study, also using ELISA to measure Aβ42, found similar results whereby, salivary Aβ42 levels were significantly higher in a cohort of 15 AD patients compared to normal controls (Sabbagh et al., 2018). Interestingly, other studies using a mass spectrometry approach did not detect amyloid beta in saliva of human patients at all (Shi et al., 2011). Other studies aimed at optimizing salivary amyloid beta measurements by adding thioflavin S to prevent its aggregation, and sodium azide to inhibit bacterial growth in saliva (Lee, Guo, Kennedy, McGeer, &

McGeer, 2017). Both of these technical steps acted to prevent sample degradation and improve sample quality and subsequent Aβ42 detection by ELISA (Lee et al., 2017). Aβ42 detection using this method found that the concentration of Aβ42 was about twice as high in AD patients (~40 pg/ml) compared to healthy controls (~20 pg/ml) (Lee et al., 2017). To test the efficiency of their method, these authors compared their in-house ELISA to the commercially available ELISA (Invitrogen) used in the early study and found that it only detected 25% of Aβ42 in the sample when compared to their method (Lee et al., 2017). These findings suggest that adding thioflavin S and sodium azide can dramatically improve salivary Aβ42 measures. This study did not report any differences in Aβ42 concentrations according to disease stage (mild, moderate, or severe) within AD patients, nor did they measure Aβ40 with their method (Lee et al., 2017). Overall, these studies had suggested that saliva testing could be a promising method for detecting AD during its critical early stages and could be useful in tracking therapeutic strategies aimed at reducing amyloid beta levels (Madav, Wairkar, & Prabhakar, 2019).

12.2.3.2 Measures of Tau in Saliva

The first study to measure tau in saliva utilized a mass spectrometry approach, which showed unequivocally that tau is present in human saliva (Shi et al., 2011). In that study, a Luminex method and a modified protocol to achieve minimal matrix effects (see below Sect. 12.3.3) were used to quantify salivary total tau (t-tau) and phosphorylated tau (p-tau) levels in 21 AD patients and 38 healthy controls. Results showed that t-tau levels were slightly decreased in AD patients compared to controls, but the difference diminished after normalizing to the total protein levels in the sample (Shi et al., 2011). There was a clear trend for increased p-tau levels in AD patients, whether or not the values were normalized to saliva total protein (Shi et al., 2011). Significant increases were also found in the p-tau/t-tau ratios in patients with AD, though the differences in t-tau and p-tau levels between patients and controls did not reach statistical significance. These findings suggest that p-tau or p-tau/t-tau ratios might be useful as a possible biomarker for AD.

Another study also quantified salivary t-tau levels using a different method, ultrasensitive Single molecule array technology, in three groups of subjects: 53 AD patients, 68 mild cognitive impairment, and 160 healthy elderly controls (Ashton et al., 2018). However, in that study, no significant differences in salivary t-tau levels were found in AD patients compared to mild cognitive impairment or healthy elderly controls (Ashton et al., 2018). Further, there was no association between salivary t-tau concentrations and clinical assessments or structural magnetic resonance imaging data (Ashton et al., 2018). Hence, more studies are needed to determine whether salivary tau could be a relevant biomarker for this disease.

12.2.3.3 Salivary Lactoferrin in AD

A growing body of literature supports the notion that immune system dysfunction plays a major role in the pathophysiology of AD (Akiyama et al., 2000; Kinney et al., 2018). Although several immune-related biomarkers have been measured in saliva, recent studies have focused on lactoferrin, an antimicrobial peptide which functions in the modulation of immune reactions and inflammatory pathways (Carro et al., 2017). In this study, both mass spectrometry and ELISA methodologies were utilized to measure lactoferrin in AD patients compared to healthy controls, and also subjects with PD, with results showing significantly reduced levels of lactoferrin in AD patients versus both control groups (Carro et al., 2017). Presumably, lower lactoferrin levels would allow for uncontrolled inflammation and immune signaling. Further, significant correlations between salivary lactoferrin and *APOE4* allele status, Mini Mental State Examination (MMSE) scores, CSF amyloid beta-42, and CSF tau were also demonstrated (Carro et al., 2017), providing convincing evidence for lactoferrin as a relevant salivary biomarker for early detection and diagnosis in AD. Importantly, lactoferrin may represent a useful salivary biomarker for other neurodegenerative diseases, many of which have been related to dysregulated immune and inflammatory mechanisms.

12.2.4 Huntington's Disease (HD): Clinical Characteristics and Pathology

HD is an inherited, progressive autosomal-dominant, neurodegenerative disorder that affects approximately one person per 10,000 people worldwide (Huntington's Disease Collaborative Research Group, 1993). It is classified as a movement disorder with the most characteristic symptom of HD being chorea, which is uncontrolled and involuntary movement of the limbs and face. In addition to motor dysfunction, which defines the clinical onset of the disease, other symptoms are commonly present. These include cognitive and psychiatric deficits, which are often detectable before the appearance of motor abnormalities. HD is typically diagnosed in one's late 30s or early 40s, with death following approximately 15 years after diagnosis, from complications such as aspiration pneumonia or cardiac failure (Heemskerk & Roos, 2012). A juvenile form of HD occurs in approximately 5% of cases (Nance & Myers, 2001; van Dijk, van der Velde, Roos, & Bruyn, 1986). Juvenile forms progress more rapidly and show more stiffness of movement rather than chorea. Seizures are also common with this form of the disease (Nance & Myers, 2001). Symptoms arise from prominent neuronal cell loss in the striatum of the brain, which is a region controlling movement and motor function. However, cell death and atrophy in cortical regions also occur.

The HD gene mutation is a CAG repeat expansion in exon 1 of the Huntington (*HTT*) gene (Huntington's Disease Collaborative Research Group, 1993). The length

of the CAG repeat expansion determines whether an individual will inherit the disease. Repeat mutations of 40 or greater lead are pathogenic, while repeat lengths <35 are generally considered to be non-pathological. In between mutations, those between 36 and 39 repeats, show unpredictable penetrance (Kremer et al., 1994; Myers et al., 1993). Although HD is a single gene disease, with genetic testing readily available, there is still substantial variability in the onset and severity of disease symptoms, even in patients with the same CAG repeat mutation (Andresen et al., 2007; Andrew et al., 1993; Wexler et al., 2004). For example, studies have shown that HD patients with CAG repeat lengths between 40 and 44 can have an age of onset that differs by up to 20 years (Andrew et al., 1993). The nature and severity of disease symptoms, as well as the course of illness, can also vary among patients, highlighting the need for biomarkers to predict and monitor these features. There are only two FDA-approved treatments available for HD (Richard & Frank, 2019; Yero & Rey, 2008). However, the most promising therapeutic approaches involve *HTT* gene silencing and knockdown technologies, which are currently in different pre-clinical and clinical stages of development.

12.2.5 The Huntingtin (Htt) Protein

The *HTT* gene encodes the huntingtin protein (Htt), a large protein consisting of 3144 amino acids that is ubiquitously expressed throughout the brain and periphery (Huntington's Disease Collaborative Research Group, 1993; Marques Sousa & Humbert, 2013). For decades, the exact function(s) of the Htt protein was elusive, but now a growing body of literature shows that Htt is an essential protein (knockout mice are embryonic-lethal) and is involved in a variety of cellular functions including gene transcription, vesicle transport, and energy metabolism (Marques Sousa & Humbert, 2013; Nasir et al., 1995). The primary pathological hallmark of HD is the formation of insoluble Htt aggregates, which are found in both the nucleus and cytoplasm, not only in neurons but other CNS and non-CNS cell types (Sathasivam et al., 1999). The expression of the polyglutamine tract in the mutated Htt protein leads to protein misfolding, and the formation of toxic soluble protein oligomers and insoluble aggregates/inclusion bodies that contributes to the disruption of many intracellular pathways (Arrasate & Finkbeiner, 2012). Although HD is primarily considered to be a CNS disease, patients with HD also exhibit a wide range of peripheral changes, including skeletal muscle dysfunction and peripheral immune system abnormalities (van der Burg, Bjorkqvist, & Brundin, 2009).

Given that levels of mutant Htt correlate with the severity of HD symptoms, and that Htt is a primary target for HD therapeutics, an obvious biomarker for HD is the disease protein itself, Htt. Accordingly, many researchers have turned to measuring Htt in CSF (Wild et al., 2015) and blood (Massai et al., 2013; Weiss et al., 2012) to assess its ability to serve as a biomarker. However, both CSF collection and blood drawing are invasive procedures, with several additional drawbacks (see Table 12.1). Further, CSF levels of Htt are known to be very low (Wild et al.,

2015) and blood measurements of Htt are confounded by the cell-type heterogeneity of blood and the requirement for large starting volumes (Table 12.1). Hence, salivary measurements of the Htt protein would be an ideal alternative. Below, advances in salivary measures of Htt, as well as other studies that have investigated salivary cortisol in HD, will be discussed.

12.2.6 Salivary Biomarkers in HD

12.2.6.1 Salivary Htt as a Biomarker for HD

Recent studies by Corey-Bloom and colleagues tested whether Htt protein was present in saliva from human subjects and whether salivary levels of Htt might represent a peripheral biomarker for HD. After showing that Htt protein was present in saliva of normal individuals using Western blot methods, saliva samples were collected from 146 HD patients and matched controls to determine if levels differed according to diagnosis (Corey-Bloom et al., 2018). Using ELISA methodology, which detected both normal and mutant forms of the Htt protein (total Htt; tHtt), these authors showed that levels of tHtt were significantly higher in saliva from HD patients versus matched, normal control subjects (Corey-Bloom et al., 2018). Further, there was a nonsignificant trend toward increased levels of tHtt in pre-symptomatic HD patients compared to normal controls, suggesting that Htt levels might accumulate prior to the onset of symptoms (Corey-Bloom et al., 2018). Similar findings were reproduced in a validation cohort of HD patients and normal controls (Corey-Bloom et al., 2018). Importantly, covariable analyses showed no gender effects on salivary tHtt, but significant positive correlations to age in both HD patients and normal controls. Levels of salivary tHtt were not found to be correlated with CAG mutation length, nor age of onset of disease symptoms (Corey-Bloom et al., 2018).

That study also assessed whether tHtt levels were correlated with the severity of disease symptoms at different stages of HD patients using various clinical scores, which included the Mental State Examination (MMSE), the Unified Huntington's Disease Rating Scale (UHDRS), and Total Functional Capacity (TFC). Results showed that salivary tHtt was significantly positively correlated to the UHDRS score and significantly negatively correlated to the TFC score, with no associations with the other measures (Corey-Bloom et al., 2018). These results indicated that salivary tHtt concentrations could have clinical relevance. One drawback of this study was the potential effect of medications, given that ~50% of the HD patients were taking antidepressants, such as Paxil, Fluoxetine, Wellbutrin, and Zoloft. How medications affect saliva production is a potential overall concern, which is addressed below. Nonetheless, a major implication of this work related to HD therapeutics, where salivary levels of Htt might serve as a biomarker to monitor the effects of Htt-lowering therapies, several of which are currently in clinical trials.

12.2.6.2 Salivary Cortisol in HD

Dysfunction of the Hypothalamic–pituitary–adrenal (HPA) axis, which traditionally is seen as the body's stress system, has been linked to learning and memory deficits in a variety of neuropsychiatric and neurodegenerative conditions, including HD (Aziz et al., 2009; Du & Pang, 2015; Hubers et al., 2015). Further, evidence supports a link between HPA dysfunction and depressive symptoms in HD, as well as AD and PD, (Aziz et al., 2009; Du & Pang, 2015; Hubers et al., 2015), however the degree to which HPA dysfunction contributes to these symptoms is unknown. Depression and cognitive deficits are among the most common of the non-motor symptoms that occur in HD, with an estimated 20–50% of HD gene carriers showing depressive symptoms and/or cognitive dysfunction (Gargiulo et al., 2009). These symptoms typically occur prior to the onset of overt motor symptoms. Several studies have measured salivary cortisol in HD patients to investigate the association between the HPA axis and presence of HD symptoms in pre-symptomatic patients and those with early-stage HD (Aziz et al., 2009; Du & Pang, 2015; Hubers et al., 2015; Shirbin et al., 2013; van Duijn et al., 2010). In one study, cortisol concentrations were measured in saliva samples from HD subjects diurnally across a single day in combination with a verbal memory performance task. It was found that the severity of motor and memory retrieval symptoms was associated with higher levels of evening cortisol (Shirbin, Chua, Churchyard, Hannan, et al., 2013). Further, this study showed a trend toward higher levels of salivary cortisol in pre-symptomatic patients suggesting that HPA dysfunction and hypercortisolism could begin much earlier that the diagnostic onset of disease but memory deficits associated with HPA axis abnormalities may only manifest once motor signs are present (Shirbin, Chua, Churchyard, Hannan, et al., 2013).

In contrast, another study showed that pre-symptomatic HD patients, who were not depressed, had significantly lower morning cortisol levels relative to early-stage HD patients and controls (Shirbin et al., 2013). Further, it was shown that the cortisol awakening response was elevated in HD patients who did show depressive-like symptoms (Shirbin, Chua, Churchyard, Lowndes, et al., 2013). Similar studies have been carried out in large animal models of HD, such as the Libechov minipig; however, no significant differences in cortisol response were detected in the HD minipigs versus normal minipigs (Schuldenzucker et al., 2018). Such studies in animal models may improve translational reliability and provide insight into stress reactivity and behavioral correlations and neurodegenerative diseases.

12.2.7 Parkinson's Disease: Clinical Characteristics and Pathology

Parkinson's disease (PD) affects approximately 1–2% of the population over the age of 60 years (Thomas & Beal, 2007). Roughly 60,000 new cases of PD present in the

USA every year and an estimated 10 million people have been diagnosed with the disease worldwide, making PD the second most common neurodegenerative disease (Thomas & Beal, 2007). PD is characterized by muscle rigidity, slowness of movement, resting tremor and other features such as impaired posture and balance and loss of autonomic movements and speech. These symptoms are progressive in nature and caused by loss of dopaminergic neurons in a small region of the midbrain known as the substantia nigra (Dauer & Przedborski, 2003). PD appears to have no strong impact on life expectancy, with most people living up to 20 years after their diagnosis.

Although most PD is sporadic in nature, there are also genetic forms. Several genes have been identified in which mutations have been shown to cause an early-onset form of the disease (Dawson & Dawson, 2003; Gasser, 2007; Kitada et al., 1998; Pramstaller et al., 2005). These findings have greatly accelerated research progress for this disease. Mutations in the alpha-synuclein gene (*SNCA*) cause autosomal-dominant PD via a toxic gain of function of the encoded mutant protein (Campelo & Silva, 2017). In contrast, other early-onset forms of PD can be caused by mutations in the genes encoding the Parkinson protein 2, E3 ubiquitin protein ligase (Parkin; *PARK2*), Parkinsonism associated deglycase (PARK7; a.k.a. DJ-1) or PTEN induced putative kinase 1 (*PINK1*) (Deng, Wang, & Jankovic, 2018; Saito, 2017; Valente et al., 2004). These mutations likely result in pathological effects due to loss-of-function mechanisms. The cause and pathogenesis of the selective loss of dopamine neurons in the substantia nigra of the brain in PD also remain unclear, but accumulating evidence implicates roles for oxidative stress and mitochondrial dysfunction (Al Shahrani, Heales, Hargreaves, & Orford, 2017). To date, no treatment has been identified that represses or slows the death of dopaminergic neurons in PD, however, there are FDA-approved options to treat symptoms, with Levodopa (also called L-dopa) being the most commonly prescribed medicine for PD.

12.2.8 PD Disease-Related Proteins: Alpha-Synuclein and DJ-1

The main pathological hallmark of adult-onset PD is the Lewy body, an insoluble inclusion body localized in the cytoplasm of neurons in the brain. There are also aggregates found in neurites, referred to as Lewy neurites. A major constituent of Lewy bodies is the aggregated form of the alpha-synuclein protein. It is therefore not surprising that mutations in the gene encoding this protein, as well as other genes in the proteasomal pathway, are observed in PD.

Alpha-synuclein is a 140 amino acid protein and member of the synuclein family. It is a neuron-specific protein that is abundantly found throughout the brain and localized in the presynaptic nerve terminals. Several functions for α-synuclein have been proposed including synaptic vesicle release and vesicle trafficking, fatty acid binding, as well as roles in neuronal survival (Dev, Hofele, Barbieri, Buchman, &

van der Putten, 2003). These roles can depend on whether the protein exists in its soluble cytosolic form, or its membrane-bound form. Alpha-synuclein pathology in PD is caused by overexpression of the *SNCA* gene resulting in increased levels of the protein in the brain. In addition to gene overexpression, other mutations in the *SNCA* gene sequence have been identified in familial PD that affects aggregation states of the alpha-synuclein protein (Narhi et al., 1999; Polymeropoulos et al., 1997). There are two major forms of alpha-synuclein aggregation that occur in PD, oligomeric aggregation, which is linked to multiple organelle dysfunction and deficits in the axonal transport system (Hsu et al., 2000), and fibrillar insoluble aggregation that leads to the formation of Lewy Bodies (Baba et al., 1998; Spillantini, Crowther, Jakes, Hasegawa, & Goedert, 1998). Both forms of alpha-synuclein have been measured in CSF and plasma from PD patients in biomarker discovery research studies (Goldman et al., 2018; Tokuda et al., 2010; Vivacqua et al., 2016).

One of the causative genes of familial Parkinson's disease, *PARK7*, encodes a 189 amino acid protein, called Parkinson protein 7, more commonly known as DJ-1. DJ-1 is thought to act as a neuroprotective antioxidant, a transcriptional regulator, and as a molecular chaperone in protein degradation (Hijioka, Inden, Yanagisawa, & Kitamura, 2017; Saito, 2017). DJ-1 is typically located in the cytoplasm of dopaminergic neurons that are not only destined to die in this disease but also can be found in the nucleus mitochondria. DJ-1 levels have been shown to be altered in the CSF and plasma from patients with PD (Lin et al., 2012).

PD lacks robust diagnostic and prognostic biomarkers; however studies on CSF and blood have been widely employed for biomarker identification studies. The diagnosis of PD currently depends on the presence of specific clinical features. However, these features appear only years after the degeneration of dopaminergic neurons. Hence, there is a growing need for biomarkers that might predict early detection of the disease as well as provide a means to monitor disease progression. Alpha-synuclein has generated enormous interest as a biomarker, not only because of its role in disease pathology but also because many therapeutic efforts have focused on reducing the aggregated form of alpha-synuclein (Goldman et al., 2018; Tokuda et al., 2010; Vivacqua et al., 2016). The identification of α-Syn and DJ-1 in human saliva, two proteins that are critically involved in both familial and sporadic PD, suggests that saliva could be a potentially important biofluid for PD biomarker studies. These will be discussed below.

12.2.9 Salivary Biomarkers in PD

12.2.9.1 Measures of Alpha-Synuclein in Saliva

In efforts to establish alpha-synuclein as a relevant salivary biomarker of PD diagnosis, studies from 2011 were the first to show the existence of alpha-synuclein in human saliva (Devic et al., 2011). They found that alpha-synuclein concentrations were significantly decreased in the saliva of PD patients as compared to healthy

controls (Devic et al., 2011). These findings were replicated in other studies using ELISA methodologies (Al-Nimer, Mshatat, & Abdulla, 2014; Vivacqua et al., 2016), one of which examined different forms of alpha-synuclein in saliva (Vivacqua et al., 2016). In that study, the authors detected a significant increase in the oligomeric form of alpha-synuclein, as well as in the oligomeric alpha-synuclein/total alpha-synuclein ratios in the saliva of PD patients compared to healthy controls (Vivacqua et al., 2016). However, levels of total alpha-synuclein were significantly decreased in the saliva of PD patients when compared to healthy controls (Vivacqua et al., 2016). In that study, it was suggested that the differences in levels of the total and oligomeric forms of the protein were due to the oligomerization of monomeric alpha-synuclein in saliva, which can lead to the lowering of the total alpha-synuclein concentrations. Importantly, within the PD population, correlations were detected between salivary alpha-synuclein levels and the severity of motor symptoms, as assessed by the Unified Parkinson's Disease Rating Scale (UPDRS), as well as other measures of disease progression, stages of illness, and cognitive impairment scores (Vivacqua et al., 2016). Overall, these findings suggest that salivary alpha-synuclein might provide a relevant means for predicting disease progression, whereby lower concentrations would reflect early stages of disease and higher concentrations indicative of a more progressive stage of illness (Vivacqua et al., 2016). A similar study found increases in oligomeric synuclein in PD patients compared to normal controls in saliva (Kang et al., 2016), as well as plasma (Pchelina et al., 2017), but no difference in total alpha-synuclein between the two groups (Kang et al., 2016). In contrast to these highly interesting findings, another study did not find differences in levels of alpha-synuclein in PD patients and controls in either saliva or plasma, nor did alpha-synuclein significantly correlate among biofluids, including CSF (Goldman et al., 2018).

12.2.9.2 DJ-1 in Saliva of PD Patients

Several studies have measured DJ-1 in saliva to assess its potential to serve as a peripheral biomarker for PD, albeit with mixed results. In one pilot study of $n = 16$ PD patients and $n = 22$ matched controls, an increase in both total protein concentration and DJ-1 concentrations were found in PD patients, but there was no difference in salivary DJ-1 after correctly for total protein concentrations in the samples (Masters, Noyce, Warner, Giovannoni, & Proctor, 2015). However, the adjusted DJ-1 levels did correlate with disease symptoms as measured by the UPDRS scores (Masters et al., 2015). In contrast, in a larger study of $n = 74$ PD patients, salivary levels of DJ-1 were higher in later stages of PD compared to early-stage patients and normal controls (Kang et al., 2014). Although in that study, salivary DJ-1 levels were not found to be correlated with UPDRS scores (Kang et al., 2014). Another study used an established Luminex assay with in-house modifications in order to achieve improved accuracy with minimal matrix effect in saliva (see below discussion on matrix effects) (Devic et al., 2011). Differences in DJ-1 levels between patients and controls did not reach statistical significance,

regardless of whether or not protein concentrations were normalized, but a trend toward increased levels of DJ-1 was observed in PD patients compared to controls (Devic et al., 2011). Again, no correlation was observed between total DJ-1 levels and UPDRS motor scores (Devic et al., 2011). In assessing DJ-1 levels specifically in buccal cells of saliva, no change was found in DJ-1 levels in PD patients compared to normal controls (Stewart et al., 2014), consistent with studies in plasma (An, Pu, Xiao, & Zhang, 2018).

12.3 Challenges and Considerations

12.3.1 Origins of Salivary Analytes

One important consideration in salivary bioscience research relating to neurological disorders is the origin of the analyte/protein being measured. While several neurodegenerative disease-related proteins are undoubtedly present in saliva, the origin of these proteins is unclear and could derive from several sources, or a combination of sources. Constituents from the blood can enter into the saliva via transcellular transport, passive intracellular diffusion, or active transport, giving credence to the notion that some salivary proteins could reflect those circulating levels in the body. Additional studies directly comparing saliva and blood levels will be necessary to test this notion.

Additionally, salivary analytes could come from the salivary glands themselves. Salivary glands are known to express several disease proteins, including the Htt protein, the amyloid beta precursor protein and tau (Conrad, Vianna, Freeman, & Davies, 2002; Marques Sousa & Humbert, 2013; Oh & Turner, 2006), which would be secreted into the whole saliva. It is also possible that the nerves innervating salivary glands could release proteins into the saliva. Parasympathetic innervation to the submandibular glands is achieved by the superior salivatory nucleus via the VIIth cranial nerve, a branch of the facial nerve, while sympathetic innervation of the salivary glands takes place via preganglionic nerves located in the intermediolateral nucleus of spinal cord (Silvers & Som, 1998). It will be critical for future investigations to explore the precise sources and contributions of these salivary biomarkers, not only for understanding the fundamental mechanisms involved in the transportation of these proteins in disease states, but also for controlling the effects of potential covariables if salivary biomarkers are to be used in the clinic.

12.3.2 Medication Considerations

Most patients with neurodegenerative diseases take several different types of medications and these could affect measurements of biomarkers in saliva. In particular, the rate of saliva secretion can be influenced by medications and other treatments.

The presence of other drugs in the body can affect accurate measures of a drug of interest. For example, antidepressant drugs, antihistamines, antipsychotics, sedatives, methyldopa, and diuretics are all known to lead to a low saliva volume or hyposalivation in many, but not all, patients. The presence of these drugs may not directly reduce saliva production, but may lead to a loss of saliva volume secondary to dehydration. This is the case, for example, of diuretics. In addition, cholinergic agents, such as those used in PD patients, can cause dry mouth or xerostomia. Moreover, patient intrinsic factors such as salivary pH and salivary protein composition vary among individuals and can be affected by the disease state itself (Kiang & Ensom, 2016).

12.3.3 Matrix Effects

Although ELISA assays designed for blood samples are often used for other biological fluids, it is important to consider the saliva matrix, which can have a confounding effect on immunoassay results. Interactions between the protein of interest and other constituents in the saliva (i.e., the saliva matrix) can result in erroneous readings, typically by affecting the binding of antibody to the protein of interest, or by altering the signal-to-noise ratio. Most matrix effects can be attributed to hydrophobic substances in the sample, such as the phospholipids and carbohydrates that make up mucins, which are abundantly present in saliva and account for its viscous nature. In addition, matrix effects can be due to the pH of the sample, protein–protein interactions, and/or differential salt and ion concentrations in the saliva. One way to assess the extent of the matrix effect is to spike-in different amounts of standards into pooled saliva samples. This has been carried out in several of the aforementioned studies with varying results. For DJ-1, the authors carefully addressed the saliva matrix effect and used a protocol that accomplished 100% recovery of DJ-1 in saliva (Devic et al., 2011). In other studies, it was found that the average recovery rate for tau proteins was between 50 and 70% (Shi et al., 2011), while that of the Htt protein was higher, at 91% (Corey-Bloom et al., 2018). Unfortunately, most studies did not mention their recovery of the measured disease protein in saliva, nor whether matrix effects were addressed.

12.4 Conclusions

There is an urgent need for the development of noninvasive and easily accessible biomarkers for neurodegenerative diseases, which are severely debilitating, not only for patients but also their family members and caregivers as well. Saliva is a biological fluid that shows significant potential for the development of noninvasive testing of disease biomarkers as summarized in this chapter. This is a particularly innovative area of research for neurodegenerative diseases, although there are still

several challenges that need to be addressed. Because relevant biomarkers might be present at very low concentrations in saliva, there is a need for more specific and sensitive analytical methods to identify and quantify these disease proteins in saliva. Finally, many of the studies on candidate biomarkers in saliva summarized in this chapter will require extensive validation in larger cohorts, but this does not diminish the exciting potential for saliva to be translated into clinical diagnostic, prognostic, and screening efforts for neurological disorders.

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