Evaluation of salivary HO-1 as a potential biomarker for Parkinson disease

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Table of Contents

Abstract (English):

Parkinson's disease (PD) is the second most common neurodegenerative disease that affects 2% of the worldwide population. PD patients face a slew of motor and non-motor symptoms which greatly affect's their quality of life including tremors, motor incoordination and even hallucinations. There are no current diagnostic biological tests available, and diagnosis can still only be confirmed during autopsy. A recent pilot study reported elevated heme oxygenase-1 (HO-1) protein levels in saliva of PD subjects. HO-1 is an inducible stress response protein important for heme degradation that has also been implicated in PD pathology. **Objectives:** To evaluate salivary HO-1 protein levels of PD subjects and assess its potential as a biomarker of PD. **Methodology:** Three hundred and seven patients were included in this study comprised of non-neurological controls (n = 162), degenerative neurological controls (n = 37), non-degenerative neurological controls (n = 33) and PD patients (n = 75). Levels of salivary HO-1 and total protein were assayed using enzyme-linked immunosorbent assay and bicinchoninic acid assay, respectively. Analyses were adjusted by age, sex, total protein and relevant comorbidities by ANCOVA and include logistic regression and receiver operating characteristic (ROC) curves analysis. **Results:** We report significantly elevated salivary total protein and HO-1 in PD subjects compared to non-neurological and non-degenerative neurological controls. ROC analyses using HO-1 in combination with covariates showed an area under the curve (AUC) of 86% and 88%, respectively. In a secondary analysis, salivary HO-1 was significantly increased in patients with neurodegenerative conditions (n = 112) relative to those without (n=195), and an AUC of 86.0%. **Conclusions**: Salivary HO-1 in concert with covariates has a potential to be a marker to distinguish patients with neurodegenerative conditions from the rest of the population.

Abstract (Français):

La maladie de Parkinson (MP) est la deuxième maladie neurodégénérative la plus fréquente avec 2% de la population mondiale atteinte. Aucun test de diagnostic n'est actuellement disponible et le diagnostic ne peut être confirmé que lors de l'autopsie. L'hème oxygénase-1 (HO-1) est une protéine de stress importante pour la dégradation de l'hème qui a été impliquée dans la pathogenèse de la MP. Une étude pilote récente a rapporté pour la première fois des niveaux élevés de protéines HO-1 dans la salive de sujets atteints de MP. **Objectifs**: Quantifier HO-1 dans la salive des sujets PD et évaluer le potentiel de cette protéine en tant que biomarqueur de la MP. **Méthodologie**: Trois cent sept patients ont été inclus dans cette étude comprenant des témoins non neurologiques (n = 162), des témoins neurologiques dégénératifs (n = 37), des témoins neurologiques non dégénératifs (n = 33) et des patients atteints de MP (n = 75). Les niveaux de HO-1 salivaire et de protéines totales ont été dosés en utilisant les dosages ELISA et BCA, respectivement. Les analyses ont été ajustées en fonction de l'âge, du sexe, des protéines totales et des comorbidités pertinentes. **Résultats**: Des taux de protéines totales salivaires et de HO-1 significativement élevés chez les sujets PD par rapport aux contrôles neurologiques non neurologiques et non dégénératifs ont été observés avec des analyses ROC utilisant HO-1 en combinaison avec des covariables montrant une aire sous la courbe de 86% et 88%, respectivement. La redéfinition de la population d'échantillonnage dans une analyse secondaire a montré que la quantité de HO-1 était significativement augmentée dans la salive des personnes souffrant d’affections neurodégénératives par rapport au groupe témoin non neurodégénératif, avec une aire sous la courbe de 86,0%. **Conclusions**: Les concentrations de HO-1 dans la salive,

de concert avec des covariables, ont le potentiel d'être un marqueur discriminatif pour les maladies neurodégénératives.

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Introduction and statement of the problem:

Neurological disorders are the leading source of disability [1]. Parkinson's disease (PD) is the second most common and fastest-growing neurodegenerative disorder in terms of prevalence, currently affecting 2% of the world's population with over 6 million people worldwide with the disease [1]. PD is characterized by the progressive loss of dopaminergic neurons in the *substantia nigra* (SN) and the formation of intracellular inclusions called Lewy bodies. While categorized as a movement disorder with motor symptoms such as bradykinesia and resting tremor, there is increasing recognition of non-motor symptoms integral to PD pathology which can occur decades prior to the onset of motor symptoms [2-4].

While increased accuracy of clinical diagnosis of PD has been enabled by the application of diagnostic criteria guidelines, there is room for further improvement to better differentiate PD from other neurological diseases with similar motor symptom manifestations [5]. The clinical diagnosis of PD still relies primarily on the presence of motor symptoms and is confirmed post-mortem. At the time of PD diagnosis, there is already substantial loss of tyrosine hydroxylase-positive neurons in the SN, indicative of a loss of dopaminergic neurons in this region, alongside reports of neuronal loss in other regions such as the putamen [6-11]. Early implementation of therapeutic strategies would enable symptom management and improvement in the quality of life of PD patients. It is therefore critical to develop biomarkers used for the detection of idiopathic PD to meet the growing need for a test that is not reliant on the evaluation of motor symptoms. In this study, I will be assessing the levels of heme oxygenase-1 (HO-1), an oxidative stress marker, in PD patients compared to healthy subjects and patients with other neurological diseases, which would address if HO-1 can be used as a potential biomarker for PD.

Background:

Parkinson's disease:

Parkinson's disease (PD), previously known as *paralysis agitans* or "shaking palsy", was first medically described in 1817 by James Parkinson as a neurological syndrome that involves not only involuntary tremors but included symptoms of rigidity and postural imbalance [2]. Since its first medical description, PD has been further characterized by cardinal motor symptoms like bradykinesia, rigidity, (4-6 Hz) resting tremor, postural imbalance, and non-motor symptoms such as rapid eye movement (REM) sleep behaviour disorder, cognitive impairment, and autonomic dysfunction [2-4, 12-14].

Prevalence

Neurological disorders are the leading sources of disability for the worldwide population [15]. Of these neurological disorders, PD is the fastest growing in terms of prevalence [15]. From 1990 to 2016, the number of people who had developed PD more than doubled from approximately 2.5 million to over 6 million people [1]. In a study that projected the prevalence of PD by the year 2040 compared to 2005 in the USA, the number of people with PD was estimated to increase in an exponential manner and double by the year 2040 when taking into account age, sex and the decline in smoking habits [16, 17]. In Canada alone, in 2013-2014, 84,000 Canadians over the age of 40 years were diagnosed with parkinsonism with a prevalence of 0.4% and an incidence rate of 55.1 per 100,000 Canadians [18]. Among Canadians over the age of 85 years the prevalence of PD reached 2.0% [18]. It was estimated that while in Canadian private households the prevalence of PD was 0.2%, the prevalence of PD in residential institutions

was 4.9% [19]. Between 1990 and 2016 the prevalence of PD has increased and this increase was suggested to be influenced by several main drivers such as increased life expectancy and the aging population [1, 18, 19]. However, the age-standardized prevalence rate of PD has still increased throughout the years meaning that the growing rate of PD is not solely dependent on our aging population [1]. The increasing global life expectancy was suggested to be due to falling tobacco use and cardiovascular disease mortality throughout the years [20]. Other factors such as declined smoking rates and increased industrialization and their effect have been linked to increased PD burden and may explain the growing prevalence of PD worldwide [17, 21].

Risk factors

It has been thought PD may result from an interplay in environmental and genetic factors, nevertheless, the etiology of PD remains unknown [22]. Other major factors that have been associated to PD risk and prevalence are age and sex [1, 18, 22-27].

Age and Sex

While the cause or trigger for PD development has yet to be identified, one of the biggest risk factors for developing idiopathic PD is accredited to aging [23-25]. The reason as to why age leads to increased risk for PD has been postulated to be connected to oxidative stress and mitochondrial dysfunction seen in PD [25, 28] (see section “Age and Oxidative Stress”). Sex is also seen as another important risk factor for PD, with PD being more prevalent in males than females [1, 26, 27]. In a French nationwide study, the prevalence male to female ratio was 1.48 [27]. Additionally, in both the French nationwide study and in a meta-analysis including sample populations in Poland, China, Italy, Spain, and the United States of America (USA) the relative incidence rate ratio of males to females was found to be 1.5, indicating that PD was more

frequent in males [26, 27]. In the French study, the male to female ratio increased by 0.05 for prevalence and by 0.14 for incidence, every 10 years of age, suggesting both age and sex as risk factors in PD [27]. The reason as to why men are at a greater risk of developing PD compared to women is unknown. It has been postulated that the "male lifestyle" such as male predominant jobs like farm work where environmental toxin exposure may be more prevalent, as well as neuroprotection by estrogen, mitochondrial dysfunction or X-linkage of genetic risk factors, may be possible reasons to explain the increasing predominance of PD in the male population [26, 29, 30]. Apart from age and sex, several environmental and genetic risk factors for PD have been identified.

Environmental risk factors

As previously mentioned, declining smoking rates and changes in smoking habits are suggested to be main drivers of increased PD prevalence worldwide. Numerous studies have shown that cigarette smoking has an inverse association with PD [31-35]. In a prospective European population-based cohort study spanning eight countries and including 715 PD cases, past cigarette smokers were shown to have a 20% decreased risk in developing PD (Hazard ratio (HR) = 0.79, 95% CI: 0.66 – 0.94), while current smokers had reduced risk by 51% compared to non-smokers (HR = 0.49, 95% CI: 0.38 – 0.63) [33]. Increased duration of smoking also decreased risk in developing PD with those with less than 20 years of smoking history having 16% reduced risk of developing PD (HR = 0.84, 95% CI: 0.67 – 1.07) while those with 30 years or greater smoking history having reduced risk of 46% compared to non-smokers [33]. In a pooled analysis of 8 case-control and 3 cohort studies (Nurses' Health Study, Health Professionals Follow-Up Study, and Honolulu-Asia Aging Study) in the USA conducted between 1960 and 2004, an inverse association

was found between PD and cigarette smoking (Odds ratio (OR) = 1.13, 95% CI, 0.93-1.37) [35]. This inverse association was also found for those smoking cigars/pipes (OR = 0.78, 95% CI: 0.58 – 1.05), and men who chewed tobacco (OR = 0.66, 95% CI: 0.43 – 1.02) compared to non-smokers [35]. In another USA case-control study including 154 PD subjects and 173 age- and sex-matched controls, passive exposure to environmental tobacco smoke, containing the same products as actively inhaled tobacco smoke, in non-smokers has been similarly reported to being inversely associated with PD (OR = 0.34, 95% CI: 0.16 – 0.73), as well as to the number of years exposed to environmental tobacco smoke by living with a daily smoker (OR = 0.86, 95% CI: 0.75 – 0.99 per year) [36]. Parental smoking during childhood exposure has also been shown to be inversely associated with the risk of PD, with subjects of two parental smokers reported to have a 27% lower risk of PD than subjects with non-parental smokers (relative rate = 0.73, 95% CI: 0.53 – 1.00) in the Nurses' Health Study and Health Professionals Follow-Up Study cohorts in the US, further implicating smoke exposure to PD risk [37]. If the association between smoking and PD are true and causal, declining smoking rates would lead to a higher incidence in PD [17].

Alongside declining smoking rates, industrialization has been a major driver in the increased prevalence of PD worldwide [1]. Exposure to by-products of growing industrialization, such as pesticides, like paraquat and rotenone, and heavy metals, like manganese, have been linked to PD [29, 30, 38-41]. Lifetime occupational exposure to pesticides increased the likelihood of having PD by two-fold in men with direct exposures through application and mixing of pesticides (OR = 2.00, 95% CI: 0.92–4.37), while those with indirect exposures had an increased likelihood of PD by about 62% (OR = 1.62; 95% CI: 1.08–2.43) [30]. Exposure to rotenone, a mitochondrial complex I inhibitor, and paraquat, an inducer of oxidative stress, have been shown

to be positively associated with PD, with the likelihood of having PD being 2.5 times greater in rotenone (OR = 2.5, 95% CI, 1.3–4.7) or paraquat exposed subjects (OR = 2.5, 95% CI, 1.4–4.7) compared to non-users [29]. Rotenone and paraquat have been used in animal models of PD, as they recapitulate many features seen in PD pathogenesis including mitochondrial dysfunction, reduced ATP production, and generation of reactive oxygen species alongside motor deficits [38, 39]. Despite rotenone and paraquat being strongly linked to PD and gradual banning of these pesticides, rotenone and paraquat are still currently in use by countries including the United States and exported to various countries by the United Kingdom [29, 30, 42, 43].

Other environmental toxins such as heavy metals have also been linked to PD. In a small Quebec case-control study including 42 PD cases and 84 controls, occupational exposure to the combination of manganese, iron and aluminum for more than 30 years was positively associated with PD (OR = 13.64, 95% CI = 1.52–76.28) [44]. Another case-control study including 144 idiopathic PD patients and 464 controls showed a positive association between PD and long term exposure of over 20 years to heavy metals such as manganese (OR = 10.61, 95% CI = 1.06 – 105.83), copper (OR = 2.49, 95% CI = 1.06 – 5.89), and a borderline association with lead (OR = 2.05, 95% CI: 0.97 – 4.31), but did not find significant association with iron, zinc or mercury [41]. A combination of exposures to heavy metals was also found to be associated with PD: combinations of lead-copper (OR = 5.24, 95%, CI = 1.59–17.21), lead-iron (OR = 2.83, 95% CI = 1.07–7.50) and iron-copper (OR = 3.69, 95% CI = 1.40–9.71) [41]. Other studies have also reported a two-to-three-fold increase in PD likelihood with lifetime lead exposure [45, 46]. There are also reports of no significant association between PD and individual heavy metal exposure for mercury, zinc, iron, lead, copper, and manganese [41, 47, 48]. Several studies had also found that

occupational or environmental exposure to heavy metals were associated with neurological syndromes including parkinsonism symptoms and cognitive deficits [49, 50]. Despite some contradicting results, exposure to individual or combinations of heavy metals has been reported to be associated with PD and parkinsonism symptoms and has been suggested as risk factors for the disease.

Genetic risk factors

While the majority of PD cases are considered idiopathic, familial PD encompasses 10% of those affected, suggesting a genetic susceptibility to the disease in these cases [51]. Of the non-familial cases, approximately 3-5% have an underlying genetic susceptibility factor [51]. Mutations in the gene encoding α-synuclein, *SNCA* or *PARK1*, are known to cause familial forms of PD and it was the first gene linked to PD [52-55]. The function of α-synuclein is unknown, however, it may modulate synaptic plasticity and dopaminergic neurotransmission under normal function [56]. Fibrillar inclusions called Lewy bodies are mainly formed of aggregation of α-synuclein and are considered a main characteristic of PD [57]. Other common mutations linked to heritable forms of PD and Lewy body formation are Parkin (*PARK2*), an E3 ubiquitin ligase involved in the degradation of misfolded or damaged proteins via the Ubiquitin-proteasome pathway, and ubiquitin carboxy-terminal hydrolase L1 (*UCHL1* or *PARK5)*, a neuron specific protein that plays a role in recycling polymeric chains of ubiquitin to its monomeric form for the Ubiquitin-proteasome pathway [58-60]. Genetic mutations in *PARK2* is one of the most common cause of autosomal recessive inheritance of PD alongside phosphatase and tensin homolog (PTEN)-induced kinase 1 (*PINK1* or *PARK6*) and Daisuke-Junko-1 (*DJ-1* or *PARK7*) mutations [61-63]. PINK1 alongside Parkin plays a role in mediating mitophagy, through accumulation of PINK1

on dysfunctional mitochondria and subsequent signalling to Parkin to ubiquinate the damaged mitochondria [64]. *DJ-1* mutations have also been linked to mitochondria dysfunction, as DJ-1 is a multifunctional protein that is involved in transcriptional regulation, oxidative stress, and chaperon, protease and mitochondrial regulation [65]. Furthermore, cases of recessive mutations are rare and cause atypical PD. This is in contrast to genetic mutations causing autosomal dominant inheritance of clinically typical PD, including *SNCA* and *LRRK2* mutations, encoding leucine-rich repeat kinase 2 (LRRK2) [61, 66, 67]. LRRK2 is a protein with both kinase and guanosine triphosphatase function and scaffolding domains. LRRK2 has been shown to co-localize with early stages of α-synuclein aggregates and it has been suggested that LRRK2 dysfunction may contribute to the formation of Lewy bodies [68]. While many gene mutations and genetic susceptibility factors have been to be linked to PD, PD cases with a genetic underlying factor are still only minor. Thus, while genetics may be a factor in PD development and pathology, most PD cases are idiopathic with much of the underlying neuropathogenesis of PD being unknown.

Comorbidities

PD patients face various other health conditions and some of these comorbidities have been linked to PD such as rheumatoid arthritis, diabetes, thyroid problems like hypothyroidism, and heart problems such as coronary artery disease and hypertension [69-74]. Conditions such as hypothyroidism, coronary artery disease, stroke, and hypertension have been associated with increased risk of PD, while rheumatoid arthritis has been reported to reduce the risk of PD [69-71, 74]. Diabetes mellitus has also been reported to be positively associated with PD (OR = 1.36, 95% CI 1.08 – 1.71) [72]. Additionally, in a prospective study following over 50,000 Finnish men

and women without PD at baseline with a mean follow up period of 18.0 years, diabetes mellitus type 2 was reported to be associated with increased risk of PD in both men (HR = 1.80, 95% CI 1.03 – 3.15) and women (HR = 1.93, 95% CI 1.05 = 2.53) [73]. While there are reports of a positive association between diabetes and PD, other studies have yielded mixed results reporting no association or inverse associations [72, 73, 75-79]. Contradicting results may result from different methodologies and sample population used in the studies.

Symptoms

Cardinal symptoms of PD include bradykinesia, muscle rigidity, 4-6 Hz rest tremor, and postural instability [12]. Measurement of motor symptoms are conducted via assessments including finger tapping, retropulsion pull test, observations of gait and hand movements, and rapid alternating movements of the hands [14]. While PD is still considered a movement disorder, there is now increasing recognition of non-motor symptoms in this disease. Non-motor symptoms such as REM sleep behaviour disorder, constipation, and altered mood and olfaction have been described as premotor symptoms of PD [4, 13, 14]. Additional non-motor symptoms are further observed with disease progressions such as autonomic dysfunction and cognitive impairment [13, 14]. The symptoms of PD can vary tremendously, from affecting mood, experiencing hallucinations, and even changes in saliva production and drooling alongside other motor symptoms [14]. Symptoms of PD can be similar or potentially overlapping with other neurological conditions. PD misdiagnosis with other degenerative parkinsonian syndromes mimicking PD like progressive supranuclear palsy and multiple system atrophy, as well as Alzheimer's disease (AD), still occurs as a result of potentially overlapping symptoms [12, 80].

Diagnosis

The complexity of PD neuropathology is also reflected in its clinical manifestations. While PD at its core centers on the presence of bradykinesia, rigidity, and resting tremor, non-motor symptoms such as constipation and REM sleep behaviour disorder can occur years to decades before the onset of motor symptoms [4]. Non-motor symptoms are not often recognised as preclinical symptoms of PD and diagnosis of PD still centers on the presence of motor symptoms [81]. The motor features of PD are used to clinically diagnose the disease with the help of diagnostic criteria including the United Kingdom Parkinson's Disease Society Brain Bank (UKPDSBB) criteria and Movement Disorder Society diagnostic criteria for PD (MDS-PD) [12]. These two criteria base PD diagnosis through three rules: i) diagnosis of a parkinsonian syndrome with the requirement of bradykinesia and at least one other sign of parkinsonian syndrome such as muscular rigidity, 4-6 Hz rest tremor, and postural instability, ii) exclusion of secondary causes of a parkinsonian syndrome, and iii) the presentation of supportive criteria for PD outweighing potential red flags [12]. While diagnosis is still primarily through observations of motor symptoms, advances in neuroimaging such as magnetic resonance imaging, [18F] fluorodopa positron emission tomography scan, single-photon emission computed tomography and transcranial ultrasound, genetics, and olfactory tests have provided some assistance in the diagnosis and staging of idiopathic PD [82-84]. However, the majority of these methods remain expensive and labour-intensive.

To further observe and gauge PD disease progression, the Hoehn and Yahr (HY) scale, the unified Parkinson's disease rating scale (UPDRS), and later the modified MDS-UPDRS, which includes the HY Scale, have been created [14]. The HY scale relies solely on motor symptoms of

progression of motor complications with disease progression. The MDS-UPDRS, is the most widely used clinical rating scale and rates 65 items in four categories, i) non-motor experiences of daily living, ii) motor experiences of daily living, iii) motor examination and iv) motor complications [14]. While the HY scale is based only on motor complications, it has been shown to also be reflective of the UPDRS and the MDS-UPDRS scaling system [85, 86].

Misdiagnosis

In cases of PD featuring the classical symptoms, the clinical diagnosis can be very accurate, with accuracy being dependent on which criteria was used and the clinician's expertise in the diagnosis of movement disorders [5]. Pooled initial diagnostic accuracy was 79.6% by experts; however, in the early stages of the disease, initial assessment accuracy can range between 26% in patients with less than 3 years of disease duration to 91% depending on the symptoms presented and the expertise of the physician in motor disorders [80]. While the pooled accuracy of clinical diagnosis increases to 83.9% during follow-up evaluations, currently, PD can only be confirmed by post-mortem examination [80].

Misdiagnosis still occurs with false-positive cases including patients with conditions more prevalent in older age such as essential tremor, progressive supranuclear palsy, multiple system atrophy, and AD [80]. The correct diagnosis of PD is key for not only prognosis but for choosing the correct therapies a patient should undergo. Misdiagnosis of PD *versus* other neurological diseases with similar or potentially overlapping symptoms and *vice versa* can lead to inappropriate administration of medication and delays in appropriate treatment that would alleviate symptoms and improve patients' quality of life. A quantitative diagnostic test, such as a diagnostic biomarker test, that does not center on qualitative observations would be extremely

beneficial in this disease in terms of therapeutics and research to be conducted alongside clinical tests for a more accurate diagnosis of PD.

Treatment

Currently, treatment of PD aims at alleviating symptoms rather than modifying the disease. In the 1960s, 3,4-dihyroxyphenylalanine (levodopa or L-DOPA), was first reported to alleviate symptoms of Parkinsonian patients [87, 88]. Since then, levodopa therapy has become the most commonly used potent drug for controlling PD symptoms such as bradykinesia, rigidity and tremor [89]. This drug can cross the blood-brain barrier and can be converted to dopamine by dopa decarboxylase and as such alleviate symptoms caused by loss of dopaminergic neurons, and lower dopamine levels [89]. Levodopa can also undergo several different pathways of conversion specifically, conversion to dopamine by dopa decarboxylase in the periphery or to an alternate product by catechol-*O*-methyl-transferase (COMT) to produce 3-*O*-methyldopa (3-OMD) in the periphery [89]. Carbidopa, a peripheral dopa decarboxylase inhibitor, can be added to levodopa therapy to enhance effectiveness by inhibition of the production of dopamine in the periphery [89, 90]. While levodopa therapy alleviates bradykinesia and other parkinsonian symptoms, side effects of the therapy such as motor fluctuations and dyskinesias frequently occur, with these complications being experienced by most patients after a 5-year duration of levodopa therapy [89]. Dopamine can be degraded into inactive metabolites to homovanillic acid by COMT and monoamine oxidase (MAO) [89]. The conversion of levodopa to 3-OMD, and accumulation of 3-OMD has been suggested to be a factor in the adverse effects of chronic levodopa therapy such as dyskinesia [91]. Combinations of levodopa with other drugs can increase the effectiveness of therapy or improve levodopa-induced dyskinesias including the use

of drugs acting as a COMT inhibitor or MAO-I inhibitor by preventing the breakdown of dopamine [89]. These therapies alongside the use of dopamine agonists all work to alleviate symptoms by enhancing intracerebral dopamine concentrations or stimulating dopamine receptors in PD subjects. Nondopaminergic drugs such as anticholinergic drugs have been shown to be effective treatments against tremors and can be used in addition to levodopa-based therapy [22, 89].

Drug-based PD therapy can have various side effects. Common adverse reactions to PD medications include nausea, vomiting, daytime drowsiness, impulse control disorders, hallucinations, and drug-induced dyskinesia [22, 89]. Upon the occurrence of disabling motor fluctuations and dyskinesia despite a continuing response from levodopa therapy, surgical treatments can be conducted [22, 89, 92, 93]. The need for surgical treatment occurs about 10 to 13 years after PD diagnosis [22]. Surgical treatments include thalamotomy, pallidotomy, and deep-brain stimulation [89, 93]. The surgical lesion procedures of thalamotomy and pallidotomy improved symptoms but included risks of irreversibly and severe side effects including dysarthria and hemiparesis [93]. This route of treatment was common prior to the levodopa era and the advent of deep-brain stimulation [93]. Currently, deep-brain stimulation is the most common surgical procedure for the treatment of advanced PD [22]. High-frequency deep-brain stimulation by implanted electrodes into specific regions of the subthalamic nucleus or globus pallidus internus have been shown to improve PD symptoms including akinesia, rigidity, tremor, and postural instability [93]. Current research into stem cell-based therapies for PD is ongoing and focuses on replenishing dopaminergic neurons in affected brain regions [94, 95].

Pathology

The etiology of neurodegeneration in PD patients is still not well understood. The onset and progression of PD are postulated to involve a wide variety of molecular pathways and their complex interplay. PD is largely idiopathic with only a minority of PD patients having a clear genetic cause for PD. Pathogenic mechanisms of idiopathic PD and neurodegeneration in this disease have been postulated to be caused by a range of factors including oxidative stress, mitochondrial dysfunction, and as previously mentioned environmental toxins.

Cardinal pathological features of PD include the degeneration of dopaminergic neurons in the substantia nigra and the formation of proteinaceous inclusions called Lewy bodies composed primarily of fibrillar α-synuclein [22, 96]. The function of α-synuclein is unknown, however, it has been postulated to play a role in modulation of synaptic plasticity, membrane stability and dopaminergic neurotransmission when in its native soluble form [56, 97, 98]. The aggregation of α-synuclein into Lewy body inclusions has been suggested to have a toxic effect in the cell through the loss of normal function and promotion of accumulation of dopamine in the cytoplasm [56, 57, 99]. Accumulation of α-synuclein has been reported in the nigrostriatal terminals and the loss of α-synuclein function in this region, as a result, may lead to oxidative stress due to the generation of hydroxyl radical via aggregation of α-synuclein mediated and production of ROS by dopamine metabolism [57, 99, 100]. A-synuclein may also be involved in the maintenance of mitochondria inner membrane integrity. A-synuclein knockout mice has been shown to present mitochondrial lipid abnormality a reduction in the electron transport chain complex I and III activity [98]. In the mitochondria of human dopaminergic neurons, accumulation of α-synuclein was shown to cause reduced mitochondrial complex I activity and increased

production of reactive oxygen species further providing evidence of the link between α-synuclein and mitochondrial function [97].

Dopaminergic neurons of the substantia nigra are particularly susceptible to oxidative stress possibly due to cytosolic dopamine metabolism producing endogenous toxins such as dopamine-quinone species, superoxide radicals, and hydrogen peroxide through auto-oxidation, degradation by MAO, and catalyzation by iron through Fenton reaction [57]. While there are physiological mechanisms to handle the production of these reactive oxygen species (ROS), in PD nigral cells undergo elevated oxidative stress indicated by elevations of OS markers [57]. ROS can promote excitotoxicity through functional alteration in protein, DNA, and lipids. OS and ROS related to dopamine metabolism have been shown to cause inactivation of the dopamine transporter, cause mitochondrial dysfunction, alteration in brain mitochondria, and dysfunction in complex I activity [101].

A major target in PD pathology is the mitochondria. The links between mitochondrial dysfunction and PD has been reported to be related to the increased ROS in the disease. The mitochondria are the main site of ROS generation within the cell via oxidative phosphorylation to generate ATP [101]. Neurons of the substantia nigra have been suggested to be susceptible to mitochondria OS. This is possibly due to elevation of intracellular calcium causing changes in mitochondrial potential and leading to production of superoxide ion radicals [102]. Calcium ion overload and ROS generation can further affect mitochondrial permeability transition, osmotic swelling and eventual loss of mitochondrial membrane integrity, and may explain the susceptibility of these neurons in PD [102]. Additional linkage of PD to mitochondrial dysfunction in relation to oxidative stress and dopamine can be seen through genetic means, with mutations

in proteins involved in mitochondrial function such as α-synuclein, parkin, DJ-1, or PINK1 been associated to familial PD [55, 60, 62, 63, 101, 103]. Mitochondrial complex I activity is reduced in idiopathic PD patient's substantia nigra pars compacta regions, further highlighting mitochondria dysfunction in PD patients. Complex I deficiencies of the mitochondrial electron transport chain are considered to be a primary source of ROS in PD [101]. This dysfunction can be induced by complex I inhibitors such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and rotenone, which is cytotoxic preferentially to DA neurons [104]. MPTP is oxidized to 1-methyl-4-phenylpryidinium and accumulates in the mitochondria where it inhibits complex I and results in decreased ATP production and increased ROS generation [104]. Rotenone, like MPTP, has been shown to increase oxidative damage and inhibit complex I leading to eventual neuronal cell death [104]. Both MPTP and rotenone have been used in various animal models of PD. MPTP and rotenone animal models display parkinsonian-like phenotypes such as bradykinesia, rigidity, akinesia postural instability, unsteady gait, tremor which responded to dopamine receptor agonist treatment [104]. Alongside these behavioural phenotypes, MPTP and rotenone PD animal models have reported features of progressive nigrostriatal neurodegeneration, loss of tyrosine hydroxylase and dopamine transporter, the formation of cytoplasmic Lewy body-like inclusions, reduced ATP generation and increased free radical production [104].

While the cause of dopaminergic neuronal degeneration is still not fully elucidated, the collection of evidence has shown that oxidative stress and mitochondrial dysfunction are major influences in PD pathological features.

Biomarkers

PD diagnosis can still only be confirmed during post-mortem observations and misdiagnosis of PD still occurs. A quantitative diagnostic test, such as a diagnostic biomarker test, as an additional tool for clinicians would thus be extremely beneficial to provide a more accurate PD diagnosis.

A biomarker can be defined as a measurable substance in a biological sample whose presence reflects a phenomenon such as alterations in cellular, biochemical, molecular, or physical states [105]. Biomarkers can be further classified as antecedent, screening, diagnostic, staging, or prognostic [105]. Sensitivity and specificity, indicating the proportion of true positives correctly identifying people with the disease and the proportion of true negatives correctly identifying people without the disease, respectively, are performance measures of the diagnostic ability of a test [106]. Receiver operating characteristic (ROC) curves gives the ability to predict an outcome by comparing sensitivity and specificity and the area under the ROC curve can be used as another performance measure of a test [106]. These performance measures to evaluate the diagnostic ability of a test are key to identify potential biomarkers.

Biomarker research in Parkinson's disease

Diagnosis of neurological disorders is largely observation-based or given after ruling out other diseases with similar symptoms following blood tests, spinal tap, imaging, and other costly measures. In the case of idiopathic PD with generally no underlying genetic cause, no diagnostic biomarker has made it into clinical use. A diagnostic biomarker for PD would be ideal as diagnosis is still only confirmed post-mortem. Research into potential biochemical markers of PD has been

performed. Studies have identified potential markers of PD including: i) protein DJ-1 in CSF, saliva, and neural-derived exosomes in plasma [107-109], ii) 8-hydroxy-2 deoxyguanosine (8-OHdG), a marker of oxidative stress, in CSF [110-112], iii) coenzyme Q10, an antioxidant important in mitochondrial electron transport chain function, in CSF and plasma [110, 113], vi) α-synuclein in CSF, neural-derived exosomes in plasma, saliva and salivary extracellular vesicles [108, 109, 114-117], and v) heme oxygenase-1 (HO-1) in serum and saliva [118, 119]. The use of a biomarker or several biomarkers in the form of a signature to diagnose a disease using easily obtainable biofluids such as saliva, would be not only less invasive for patients but also a less costly procedure.

Salivary Biomarkers

Saliva has several advantages in comparison to other biofluids for biomarker research. Firstly, the procedure of acquiring saliva from patients is non-invasive in comparison to blood sample collection, in which difficulties can occur if the patient has small or fragile veins or compared to lumbar puncture for CSF collection. Secondly, personnel training for saliva specimen collection is not extensive, making it a biofluid with high potential for screening and analysis. While there are advantageous attributes, the use of saliva as a diagnostic fluid is uncommon due to lower levels of analytes in saliva in comparison to other biological fluids, with analytes such as immunoglobulin concentrations in serum detectable at mg/ml while whole saliva immunoglobulin concentrations ranges in the ug/ml range [120]. Despite this, the search for salivary biomarkers of disease and research into their potential as a diagnostic tool is important to alleviate patient discomfort by providing a non-invasive method of disease screening.

Saliva is generated within the sublingual, submandibular, and parotid salivary glands under the direct parasympathetic innervation of the glossopharyngeal and facial nerve [121]. The development of new technologies has allowed for research into potential biomarkers of disease in saliva, and investigations into its possible use as a diagnostic biofluid. Circulating potential biomarkers have been identified in salivary secretions for various diseases including PD, AD, Sjögren's syndrome, and oral cancer [107, 114-116, 118, 122-125]. Salivary glands are highly permeable and surrounded by capillaries allowing for the potential exchange of blood-based molecules [126]. Altered composition of salivary secretion has been suggested to be caused by the absorption of substances into the salivary gland from peripheral blood as well as from nerves innervating the salivary glands [126, 127]. Proteins have also been proposed to enter saliva via extracellular vesicles. Neural-derived extracellular vesicles including exosomes have been derived from various biofluids such as plasma and saliva, and have been found to have altered levels of potential PD biomarkers including α-synuclein and DJ-1 [109, 114]. As such, saliva may be representative of the physical well-being of an individual and can indicate the presence of disease [124].

Importance of normalization

Saliva sample collection from patients is simple and non-invasive, but like with many biofluids, salivary content can be highly variable between patients. This variability between patients highlights the importance of normalization of results or adjustments based on potential confounders when investigating potential biomarkers. Sample variation in protein and analyte concentrations can be attributed to salivary flow rate, patient physical state such as hydration levels, and whether the individual had eaten prior to the collection [128]. Normalization can be

done by comparing to total protein or other analytes in the biofluid which are expressed in high and stable quantities; for example, urine biomarkers are typically normalized to urinary creatine levels [129]. However, saliva does not have a gold standard for normalization. To account for variability in patient samples, adjustments to salivary total protein have been used to normalize analyte concentration although this is not consistently done in all salivary biomarker studies [107, 128]. Sample normalization is important to determine if total protein concentration is a factor in changes in analyte concentration and to consider the sample-to-sample variation between patients in a study. In addition to the use of normalization, adjustments of potential confounders such as comorbidities and collection time, to account for potential circadian rhythm influences, would be appropriate to minimize variability [128].

Aging and oxidative stress

Aging is a major risk factor for PD development, and it has been postulated that this connection is influenced in part by age-dependent oxidative stress and mitochondria damage and dysfunction [130].

Oxidative stress has been implicated to have a significant role in normal aging through the free radical theory of aging [131]. The free radical theory is based on the accumulation of free radicals mediating increased oxidative damage to various tissues and cells, and to the mitochondria [131]. The free radical theory of aging was later redefined to the mitochondrial theory of aging, in which it was suggested that the mitochondria is the major target of free radical damage which leads to human aging [130].

Numerous studies have implicated oxidative damage and mitochondrial damage to altered life expectancy in animal models and human [131-135]. Various literature has provided evidence of decreased mitochondrial complex I and complex IV activity in an age-dependent manner in animal models, as well as age-dependent decrease in mitochondrial ATP generation by 8% in humans per decade during normal aging [130, 132, 134]. It was also shown that there is an age-dependent accumulation of mitochondria DNA mutations [133, 135]. These mutations have been reported to accelerate aging and shorter lifespan in mice, with cytochrome oxidase activity being correlated negatively with this increased mutational burden [133]. Furthermore, 8-hydroxy-2'-deoxyguanosine, which is produced following free radical oxidation of deoxyguanosine, was shown to be elevated in an age-dependent manner in human brain tissue as well as in plasma and CSF of human subjects [136, 137]. In addition, an age-related accumulation of oxidative damage to DNA in human brain tissue was observed by measurements of 8-hydroxy-2'-deoxyguanosine, particularly to mitochondrial DNA; lending additional credibility to mitochondrial damage due to oxidative stress as a potential factor in normal aging [137].

Heme oxygenase-1

Heme oxygenase (HO) was first discovered by Tenhunen *et al*. in 1968 as a Nicotinamide adenine dinucleotide phosphate (NADPH)-dependent microsomal enzyme that metabolizes heme to biliverdin, carbon monoxide (CO), and free ferrous iron (Fe^{2+}) [138, 139]. In mammals, there are two main HO isoforms, namely heme oxygenase-1 (HO-1, 32kDa) and heme oxygenase-2 (HO-2, 36kDa) [140-142]. Though the two main isoforms have a similar function in heme degradation, HO-2 has a constitutive expression, whereas HO-1 is highly inducible. Apart from expression levels, the two isoforms exhibit 43% amino acid sequence homology and are distinct

with regards to molecular weight, tissue distribution, regulation, activity level at normal physiological state, and antigenicity [143]. Due to the highly inducible nature of HO-1, I will be focusing on this isoform in this study.

Regulation and physiology

Under normal physiological conditions, HO-1 is expressed in the spleen and gastrointestinal tract, and low HO-1 expression can be found in the brain and other tissues [144-147]. As an inducible enzyme, the HO-1 gene, *HMOX1,* has regulatory regions that allow it to respond to numerous stressors such as oxidative and inflammatory stimuli, including increased levels of heme, dopamine, and nitric oxide [148-150]. *HMOX1* induction is mainly regulated by the transcription factor, nuclear factor erythroid 2-related factor 2 (Nrf2), and its suppression is mediated by transcription regulator protein BTB Domain and CNC homolog 1 (BACH1) [148, 151, 152]. Alongside regulation of the heme metabolism through induction of *HMOX1,* Nrf2 functions also as a chief regulator of transcription for a variety of antioxidant genes, including genes involved in the glutathione and thioredoxin systems and NADPH regeneration [153, 154]. HO-1 is anchored to the endoplasmic reticulum (ER) membrane by its transmembrane sequence (TMS) located at the C-terminus with its catalytic side facing the cytosol, where it oxidizes heme, with the help of NADPH cytochrome p450 reductase, to produce biliverdin, CO, and Fe^{2+} [138, 139, 155]. The heme degradation products have been shown to have both positive and negative effects on cell state in terms of cell survival and metabolism during bouts of oxidative stress [28].

Neuroprotective and neurodystrophic effects of HO-1:

The induction of *HMOX1* after neural insults, such as ischemic stroke, may mediate neuroprotection against oxidative stress potentially through the generation of the antioxidants,

biliverdin and bilirubin, following conversion by biliverdin reductase [156, 157]. A reduction in glutamate-induced neuronal cell death was observed in isolated neurons of transgenic mice overexpressing HO-1 [158]. The importance of HO-1 was reinforced with peptide inhibition of HO-1 exacerbating glutamate-induced neurotoxicity with a 60% neuronal loss [158]. *HMOX1* induction in neurons and astrocytes has also been linked to neuroprotection against β-amyloid and H_2O_2 induced neurotoxicity and cerebral ischemia [156, 158-161]. During acute oxidative stress-induced neurotoxicity HO-1 has cytoprotective effects; however, chronic expression of HO-1 has been proposed to be detrimental to cell health and survival [28].

The dual nature of HO-1 is exemplified by chronic astrocyte HO-1 overexpression in a transgenic GFAP.HMOX1 mice model which displayed schizophrenia- and parkinsonism-like behaviour [162]. Alongside producing biliverdin, heme degradation by HO-1 produces iron (Fe^{2+}) and CO which have been suggested to be detrimental to cell metabolism and survival during chronic exposure, especially in glial cells [163, 164]. Overexpression of human HO-1 in rat astroglia through transient transfection has been reported to facilitate iron sequestration in the mitochondrial compartment causing morphological alteration such as disrupted cristae, and spherical inclusions resembling human corpora amylacea [163]. These displays of mitochondrial morphological changes were negated through treatment with tin mesoporphyrin, an HO inhibitor, suggesting the morphological alterations are dependent on canonical HO activity [163]. Oxidative mitochondrial damage as a result of glial HO-1 overexpression led to mitochondrial degeneration and bioenergetic failure from subsequent ATP depletion, increased cell death, and further exacerbation of oxidative stress [164]. With association with both positive and negative

effects on cell metabolism and survival, HO-1 has been connected to a variety of degenerative and non-degenerative neurological disorders.

Involvement in neurological conditions

HO-1 expression has been implicated in PD and various other neurological conditions such as AD, mild cognitive impairment (MCI), multiple sclerosis, epilepsy and seizures, stroke, and essential tremor.

HO-1 in Parkinson's disease

Considerable evidence from various laboratories over the last few decades has implicated HO-1 in the pathogenesis of PD in both humans and animal models [147, 162, 165-167]. Alongside hallmarks of PD pathogenesis, such as the formation of Lewy body inclusions and loss of dopaminergic neurons, PD patients also exhibit other core pathological features such as increased oxidative stress, excessive iron levels, mitochondria dysfunction, and macroautophagy in neurons of the SN [168-170]. These features were mirrored during sustained upregulation of HO-1 in *in vitro* experiments using rat astroglial cells [165].

In the post-mortem PD brain, HO-1 immunoreactivity has been detected in neuromelanin-containing dopaminergic neurons, particularly decorating Lewy bodies [147, 166]. Furthermore, a higher proportion of GFAP-positive astrocytes in the SN of PD patients' post-mortem brain was shown to be HO-1 immunoreactive in comparison to control brain specimens [147]. As HO-1 presence is a sign of oxidative stress, it is thought that PD patients experience chronic oxidative stress contributing to the clinical phenotype. The possible contribution of oxidative stress to PD phenotype is further validated by a GFAP.HMOX1 transgenic mice models

exhibiting Parkinson-like phenotypes including neurodegeneration, hypodopaminergia, altered gait, locomotor incoordination, and reduced olfaction during chronic human HO-1 induction in astrocytes from mid-to-late life [162, 167]. The GFAP.HMOX1 model also features PD-like pathological features such as increased oxidative stress, excessive iron levels, mitochondria dysfunction, and macroautophagy in neurons of the SN [162, 167]. The expression of HO-1 in several peripheral biofluids of PD patients has been investigated with reports of elevated serum and salivary HO-1 concentrations in PD patients in comparison to healthy individuals, although no observable difference was seen in the plasma [118, 119, 171].

HO-1 in Alzheimer's disease and mild cognitive impairment

AD is a progressive neurodegenerative disease characterized by dementia and the accumulation of intracellular inclusions of neurofibrillary tangles and extracellular deposits of amyloid senile plaques in regions of the basal forebrain, hippocampus, and association cortices [172]. AD also features gliosis, pathological iron deposition, mitochondrial insufficiency, and oxidative stress [172, 173]. Immunoreactive HO-1 protein is detectable in the post-mortem AD brain within astrocytes, neurons, senile plaques, and neurofibrillary tangles [145, 174]. Expression of HO-1 by induction of the HMOX1 gene has been reported to occur early in AD pathology with overexpression of HO-1 protein in patients afflicted with MCI, a frequent harbinger of AD [175]. MCI patients are those who experience cognitive decline that is more than expected of normal aging but not yet at the level of dementia. Approximately 31% of MCI patients progress to full AD within 10 years of observation [176]. Contrary to the increased HO-1 detected in AD post-mortem brains, HO-1 protein or mRNA levels were reported to be suppressed in AD biofluids (cerebrospinal fluid (CSF) and plasma), and no observable difference was reported in

the serum of AD patients [171]. This report of suppressed HO-1 levels in AD CSF and plasma were attributed to the presence of alpha-1-antitrypsin, an HO-1 suppressor factor [177].

HO-1 in multiple sclerosis:

Multiple sclerosis (MS) is an autoimmune-mediated disease that affects the central nervous system through the demyelination of nerve cells [178]. The damage to nerve cells leads to a slew of symptoms including numbness or weakness of the limbs, tremor, loss of coordination, visual and other sensory deficits, impaired bowel or bladder functions, and unsteady gate [179, 180]. Similar to post-mortem AD and PD brains, an increased proportion of GFAP-positive astrocytes immunoreactive for HO-1 was detected in MS patient spinal white matter relative to controls [181]. Expression of HO-1 has also been seen in MS brain oligodendrocytes paralleled by severe morphological damage due to oxidative stress leading to mitochondrial dysfunction and subsequent apoptotic cell death [182]. HO-1 has been detected in the peripheral tissues and biofluids of MS patients [178, 183]. Similar to the increased immunoreactivity and upregulation of HO-1 in MS post-mortem brain, increased HO-1 protein alongside other oxidative stress markers were significantly elevated in CSF and plasma of MS patients compared to controls [178]. This is in contrast to reduced HO-1 expression observed in peripheral blood mononuclear cells of MS patients [183].

HO-1 in seizures and epilepsy:

Seizures are the manifestations of abnormal neuronal discharges in the cerebral cortex occurring due to a sudden imbalance between excitatory and inhibitory signals [184]. Seizures induce a severe increase in blood flow to the brain which helps to ameliorate the excitotoxic brain damage such as vasculature damage, which could lead to permanent neuronal impairment

[184]. Reactive oxygen species are large contributors to cerebral vascular endothelial cell damage. Investigation on the contribution of ROS to seizure-induced vascular damage and the mechanism of vasoprotection in bicuculline models of epileptic seizures showed seizures increased ROS in cerebral vessels and cortical astrocytes [185]. Seizures in a kindling rat model, showed induction of oxidative stress via expression of Nrf2 and HO-1 at both the protein and mRNA level in the hippocampus [186]. Furthermore, HO inhibitor, tin protoporphyrin, potentiated the seizure-induced increase in ROS, while HO-1 inducer cobalt protoporphyrin blocked this increase of ROS and provided endogenous protection against seizure-induced vascular injury [184, 185]. While there is limited literature on the subject, these findings suggest links between the heme oxygenase system and seizures.

HO-1 in Stroke, Neuropathic pain and Essential Tremor:

The relationships of HO-1 with other neurological conditions such as stroke, essential tremor, and neuropathic pain have been investigated. The association between higher serum bilirubin, a product of the heme degradation pathway, and reduced stroke prevalence, and improved stroke outcome has been reported by multiple laboratories throughout the years [187-189]. As HO-1 leads to the production of bilirubin, the relationship between HO-1 and stroke was further explored. Higher serum HO-1 levels and lower serum bilirubin in stroke patients compared to transient ischemic attack patients and patients without symptoms of intracerebral hemorrhage have been observed suggesting an underlying link between HO-1, bilirubin, and stroke [190, 191]. The relationship between HO-1 and stroke is further validated through several animal model studies [156, 160, 192]. Stroke models made through intra-arterial suture occlusions of the middle cerebral artery in transgenic mice showed that overexpression of HO-1

resulted in significantly reduced infarct volumes, while HO-1 knockout mice had significantly larger infarct size compared to wild-type control counterparts [160, 192]. Another transgenic mouse model, in which overexpression of human HO-1 in astrocytes can be induced, have shown that astrocyte HO-1 reduced mortality and improved the outcome after collagenase-induced intracerebral hemorrhage compared to wild-type control mice [156].

The heme oxygenase family has also been implicated in neuropathic and incisional pain in several animal models [193, 194]. Rat neuropathic models through unilateral ligation of L5 and L6 nerve roots and incisional pain models were shown to have displayed mechanical allodynia and thermal hyperalgesia [194]. These pain behaviours were displayed alongside increased spinal cord HO activity and were reversed by systemic administration of zinc protoporphyrin, an HO inhibitor [194]. The results suggested a role for HO in nociceptive signaling in both neuropathic and incisional models of pain [194]. HO-1 has also been implicated in diabetic neuropathy [193]. In contrast to findings of inhibition of HO-1 alleviating mechanical allodynia and thermal hyperalgesia in the rat neuropathic model, HO-1 induction reduced streptozotocin-induced diabetic neuropathy in mice [193]. Alongside these findings, induction of HO-1 by cobalt protoporphyrin enhanced antinociceptive effects of morphine, while these effects were reversed by HO inhibitor zinc protoporphyrin [193]. These findings suggest the heme oxygenase family play a role in neuropathic pain and incisional pain in various animal models.

PD false-positive cases include patients with essential tremor (ET) [80]. ET and PD share overlapping clinical, neurophysiological, and neuroimaging features that make it difficult to distinguish ET from PD [195]. The relationship and implication of HO-1 in essential tremor has not been fully elucidated. However, an association between an allelic variant *HMOX1* rs2021746T

and the risk for essential tremor has been observed with decreased risk for essential tremor in individuals carrying the HMOX1 rs2021746T allele variant [196].

With HO-1 as a marker of oxidative stress and links to PD and several neurological diseases seen in peripheral tissue and biofluids, investigations into HO-1 as a potential biomarker of diseases with non-neurological healthy controls and neurological controls is both important and relevant to determine the specificity and to address potential biofluid-specific discrepancies.

Rationale for study, primary and secondary aims:

The development of saliva as a diagnostic tool for neurodegenerative diseases, such as PD, would be advantageous as it is easy and non-invasive to collect unlike other biofluids used in clinical practices. This project was conducted to investigate salivary HO-1 levels in PD patients against non-neurological and neurological controls subdivided into two groups (non-degenerative and degenerative neurological controls). This would allow us to determine if HO-1 can be used as a potential biomarker for PD. Investigating the difference between salivary HO-1 levels in PD against neurological controls, degenerative and non-degenerative neurological controls would give a foundation for future research for HO-1 as a tool for differential diagnosis. The development of such a biomarker would help give a clear PD diagnosis to patients and enable correct treatment regimen; as well as lowering misdiagnosis of PD in the elderly population. Reported elevated levels of total salivary protein in PD patients alongside the fact that PD patients suffer from autonomic dysfunction renders it important to evaluate and account for total protein as a potential confounder when assessing the salivary HO-1 protein concentration in the subjects.

Primary aim:

The primary aim is to evaluate salivary HO-1 protein levels in PD patients compared to i) non-neurological (healthy) controls, ii) non-degenerative neurological controls and iii) degenerative neurological controls.

Secondary aim:

The secondary aim of this project is to i) evaluate salivary HO-1 protein levels in neurodegenerative conditions compared to non-neurodegenerative control group, and ii) evaluate the use of salivary HO-1 as a potential diagnostic biomarker.

A successful outcome of these aims would further validate HO-1 as a potential biomarker for PD or neurodegenerative conditions and the use of saliva as a diagnostic biofluid.

Methodology:

Study design and population.

This case-control study was approved by the Research Ethics Committee of the Jewish General Hospital (JGH; Montreal, Canada, No. 2019-1220). From the JGH salivary biobank, a total of 307 subjects were identified to matching our enrolment criteria and included in this study comprising of 75 PD subjects, 162 healthy control subjects, 37 degenerative neurological control subjects (19 AD and 18 MCI), and 33 non-degenerative neurological controls (13 multiple sclerosis, 12 epilepsy, 3 essential tremor, 3 stroke, and 2 nerve pain patients). All participants provided written informed consent. Subjects diagnosed with idiopathic PD and other neurological disorders were recruited from the Department of Neurology at the JGH. Idiopathic PD patients fulfilled UK Parkinson's Disease Society Brain Bank diagnostic criteria and the diagnosis of subjects with PD and other neurological conditions were corroborated by serial clinical evaluations. Non-neurological controls were recruited from the Departments of Medicine, Dentistry, and Ophthalmology at the JGH. Exclusion criteria for all groups included cigarette smoking within the past year, history of oral cancer, active systemic inflammatory disease, current alcoholism and drug abuse, and evidence of atypical or familial parkinsonism.

Clinical and demographic data:

Demographics and medical histories were taken via medical history questionnaire on all subjects recruited to the JGH salivary biobank. All PD subjects underwent complete neurological examinations by a JGH neurologist, with HY scores to indicate disease progression of PD patients provided in medical charts.

Exposure to medication type and dosage varied between PD patients. Doses of pro-dopamine medications were provided in medical charts of 70 PD patients and were converted to levodopa equivalent daily dose (LEDD) to be used for evaluation of the potential effects of levodopa medication (https://www.parkinsonsmeasurement.org/toolBox/levodopaEquivalentDose.htm).

Saliva Collection and processing:

Unstimulated whole saliva samples were collected from each patient by passive drooling into sterile 50 ml conical centrifuge tubes to preserve the natural chemical composition of the sample. Saliva was collected before or after participants received their medical examinations/follow-ups and at least 30 minutes after food or liquid ingestion. Samples were kept at 4°C for a maximum of 3 hours prior to processing. The saliva was centrifuged at 10 000 rpm (7,826 x g) for 20 min at 4°C to reduce viscosity and remove food debris. The supernatant was then aliquoted and stored at -80 °C until further analysis. Prior to analysis, protease inhibitors were added upon the first thaw and all samples were re-processed through a high-speed centrifugation step at 10 000 rpm (7,826 x g) for 20 min at 4°C to reduce sample viscosity.

Quantification of salivary HO-1 and total protein levels.

HO-1 levels were measures in unstimulated saliva using human HO-1 sandwich enzyme-linked immunosorbent assay (ELISA) kit according to manufacturer instructions (Abbexa, Cat. No. abx252635) that were optimised for the detection of native samples. The detection range of the HO-1 ELISA kit is 0.156-10.00 ng/ml with a sensitivity of 0.10 ng/ml. Intra-assay and inter-assay variability was < 10% according to the commercial kit's specification. Unstimulated whole saliva

was diluted 1:10 with standard dilution buffer prior to dispensing into 96-well plates in duplicate. Samples were incubated for 15 minutes at 37°C in the dark with 3,3',5,5'-tetramethylbenzidine for visualisation. Salivary total protein concentrations were determined using bicinchoninic acid (BCA) assay (Bio-Rad DC TM Protein Assay, Cat. No. 500). Optical density readout for the salivary HO-1 ELISAs and bicinchoninic acid assays were assessed at 450 nm and 750 nm wavelengths, respectively, using a Bio-Rad Benchmark Plus™ microplate spectrophotometer.

Statistical analysis:

Crude analysis consisted of analysis of variance (ANOVA) to assess the difference in the level of salivary HO-1 and total protein concentration between groups without adjusting for age, sex, and relevant comorbidities. The potential correlation between total salivary protein and salivary HO-1 was assessed using Pearson correlation. Analysis of covariance (ANCOVA) was used to adjust for age, sex, relevant comorbidities (i.e. arthritis, thyroid problems, heart problems, and diabetes), and salivary total protein concentration. Univariate and multivariate logistic regression analysis and receiver operative characteristic (ROC) curves were generated to assess how well salivary HO-1 and other predictors can distinguish cases and controls from each other. Kolmogorox-Smirnov test and observation of quantile-quantile (Q-Q) plot was used to evaluate the normality and distribution of the sample population. Levene's test was used to evaluate the homogeneity of variance assumption.

Results:

Demographic and Comorbidity Distribution between study groups.

A total of 307 subjects' saliva samples was identified to match inclusion criteria in the Jewish General Hospital saliva biobank and included in this study. The sample population comprises of 162 (53%) non-neurological controls, 37 (12%) degenerative neurological controls, 33 (11%) non-degenerative neurological controls, and 75 (24%) PD subjects. Of these patients 162 (53%) were female and 145 (47%) were male. Table 1 shows the demographics and comorbidity distribution of these study groups.

TABLE 1. Demographic and comorbidity distribution of study groups

Groups	Non-NeuroCtl		Degen. NeuroCtl		Non-Degen. NeuroCtl		PD	
n	162		37		33		75	
Age (Mean(SD))	62	12	80	6	61	14	73	11
Female (%)	99	61%	21	57%	24	73%	18	24%
Male (%)	63	39%	16	43%	9	27%	57	76%
Arthritis (%)	43	27%	12	32%	10	27%	30	40%
No Arthritis (%)	119	74%	25	68%	23	73%	45	60%
Thyroid Problems (%)	29	18%	4	11%	10	30%	16	21%
No-Thyroid Problems (%)	133	82%	33	89%	23	70%	59	79%
Heart Problems (%)	9	6%	9	24%	3	9%	12	16%
No-Heart Problems (%)	153	94%	28	76%	30	91%	63	84%
Diabetes (%)	19	12%	6	16%	3	9%	4	5.3
No Diabetes (%)	143	88%	31	84%	30	91%	71	95%

Unadjusted salivary HO-1 concentration:

The HO-1 results were determined to have a normal distribution of residuals based on the observance of the Q-Q plot and Kolmogorov-Smirnoff test (P = 0.11) (Figure 1A). The data also passed the Levene's test for equal variance (P = 0.07). As the results were normally distributed, ANOVA was conducted for the crude analysis (Figure 1B). Unadjusted mean HO-1 protein concentrations for non-neurological controls was 55.79 ng/ml (95% confidence interval (CI): 51.17 – 59.40 ng/ml), 65.69 ng/ml (95% CI: 58.12 – 73.26 ng/ml) for degenerative neurological controls, 50.55 ng/ml (95% CI: 42.53 – 58.56 ng/ml) for non-degenerative neurological controls, and 63.56 ng/ml (95% CI: 58.24 – 68.88 ng/ml) for PD patients (Table 3). ANOVA analysis showed a significant increase in salivary mean HO-1 in the PD group in comparison to non-neurological controls (P = 0.02) and non-degenerative neurological controls (P < 0.01). No significant difference was found between PD and degenerative neurological controls (P = 0.65).

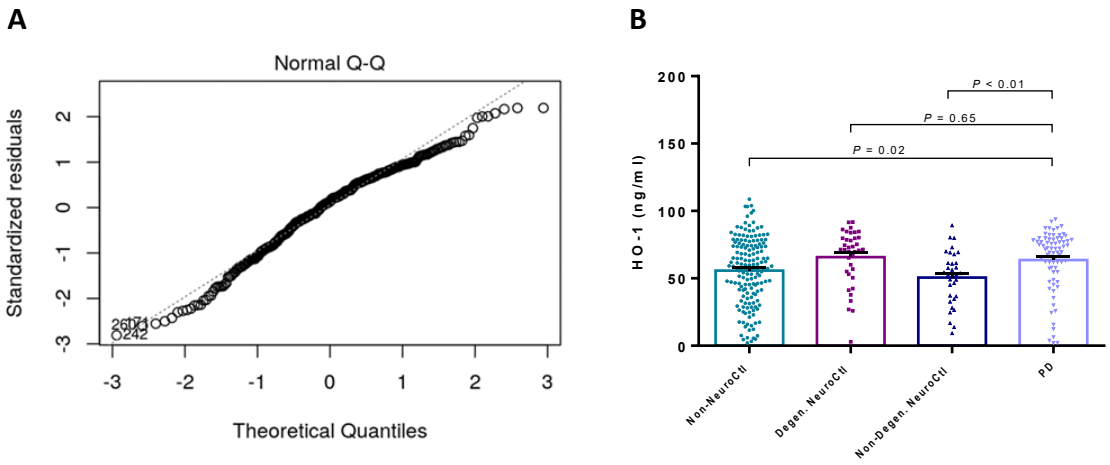

Figure 1. *Unadjusted salivary HO-1 protein levels of groups.* A) Normal distribution was visually assessed by Q-Q plot of salivary HO-1 protein level residuals and threshold 45°-line y=x. B) Unadjusted unstimulated whole saliva mean HO-1 protein levels measured by sandwich ELISA of non-neurological control group (n = 162), degenerative neurological controls (n = 37), and non-degenerative neurological controls (n = 33) and PD group (n = 75). Statistical analysis performed using ANOVA with $\alpha = 0.05$, error bars indicate the mean with SEM.

Salivary total protein and relation to HO-1:

Pearson correlation was used to determine the relationship between salivary HO-1 and salivary total protein. No significant correlation between salivary HO-1 and total protein was observed (r = - 0.74, P = 0.20, Figure 2A).

Mean salivary total protein for non-neurological control group was 2.81 mg/ml (95% CI: 2.60 – 3.03 mg/ml), 3.33 mg/ml (95% CI: 2.79 – 3.86 mg/ml) for degenerative neurological controls, 3.31 mg/ml (95% CI: 2.84 – 3.78 mg/ml) for non-degenerative neurological controls and 4.01 mg/ml (95% CI: 3.55 – 4.48 mg/ml) for PD subjects (Table 2). Contrastingly from results of the Pearson correlation, ANOVA analysis on salivary total protein showed significant elevated protein level in the PD subjects compared to non-neurological (P < 0.01) and non-degenerative neurological controls (P = 0.04, Figure 2B). Higher salivary mean total protein levels were also observed in PD subjects compared to degenerative neurological controls although this was considered a trend and not statistically significant (P = 0.09, Figure 2B).

Table 2. Salivary total protein levels

		Unadjusted Analysis		
Group	**n**	**Mean (mg/ml)**	**95% CI**	**P Value**
Non-neurological controls	162	2.81	2.60 - 3.03	<0.01
Degenerative neurological controls	37	3.33	2.79 - 3.86	0.09
Non-degenerative neurological controls	33	3.31	2.84 - 3.78	0.04
PD subjects	75	4.01	3.55 - 4.48	

PD subjects as reference group.

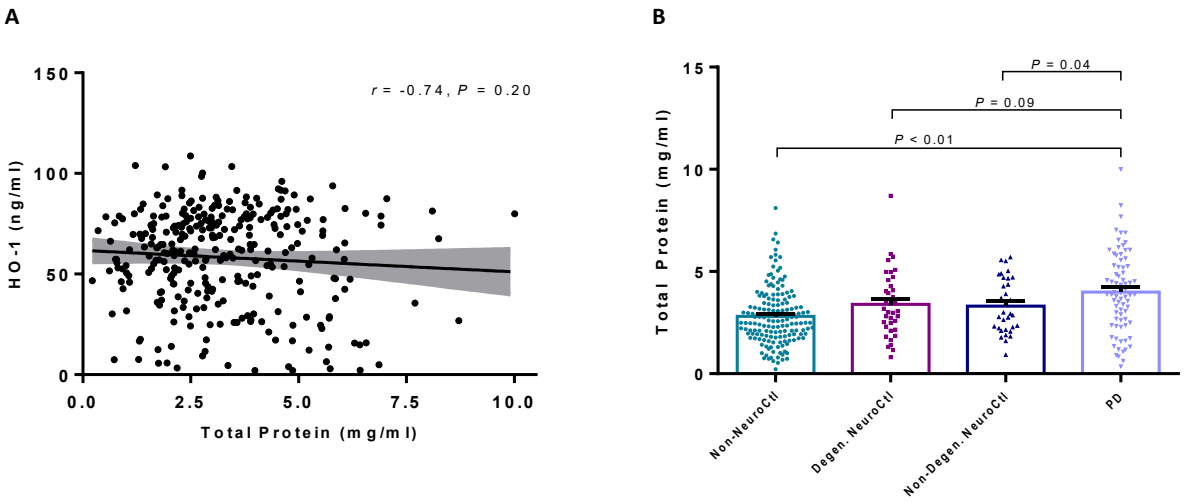

Figure 2. *Pearson correlation of salivary total protein and HO-1 and salivary total protein between groups.* A) Non-significant correlation between salivary total protein and salivary HO-1 of study's sample population. B) Unadjusted mean salivary total protein measured by BCA assay of non-neurological control group (n = 162), degenerative neurological controls (n = 37), and non-degenerative neurological controls (n = 33) and PD group (n = 75). Statistical analysis performed using ANOVA with $\alpha = 0.05$, error bars indicate the mean with SEM.

Adjusted salivary HO-1 analysis:

Salivary HO-1 levels were adjusted by the potential covariates age, sex, salivary total protein, and comorbidities such as arthritis, thyroid problems, heart problems and diabetes (Table 3). ANCOVA showed a difference between groups ($P < 0.01$). Similar to results from the crude analysis, salivary HO-1 levels was significantly elevated in PD group (63.40 ng/ml, 95% CI: 55.75 – 71.05 ng/ml) relative to non-neurological controls (55.12 ng/ml, 95% CI: 48.75 – 61.51 ng/ml, $P = 0.03$) and non-degenerative neurological controls (51.61 ng/ml, 95% CI: 41.19 – 61.03 ng/ml, $P = 0.03$) after adjustments (Table 3). Again, no statistically significant difference was found comparing PD to degenerative neurological controls (60.93 ng/ml, 95% CI: 51.25 – 70.62 ng/ml, $P = 0.63$). Table 4 shows the relationship between the covariates, age, sex, total protein, and relevant comorbidities and salivary HO-1 in this ANCOVA analysis. Salivary total protein was significantly associated in this model ($P = 0.02$). No association was found between salivary HO-1 and age, sex or comorbidities, however, a borderline association was observed between salivary HO-1 and age ($P = 0.08$) and thyroid problems ($P = 0.08$).

Table 3. Salivary HO-1 levels (adjusted by age, sex, total protein and comorbidities)

		Unadjusted Analysis			Adjusted by Age, Sex, and Comorbidities		
Group	**n**	**Mean (ng/ml)**	**95% CI**	**P Value**	**Mean (ng/ml)**	**95% CI**	**P Value**
Non-neurological controls	162	55.79	52.17 - 59.41	0.02	55.13	48.75 - 61.51	0.03
Degenerative neurological controls	37	65.69	58.12 - 73.26	0.65	60.93	51.25 - 70.62	0.63
Non-degenerative neurological controls	33	50.55	42.53 - 58.56	<0.01	51.61	42.19 - 61.03	0.03
PD subjects*	75	63.56	58.24 - 68.88		63.40	55.75 - 71.05	

*PD subjects as reference group.

TABLE 4. Primary analysis: ANVOCA covariates' relationship to HO-1 of primary analysis groups

Covariates	*P* Value
Protein	0.02
Age	0.08
Sex	0.51
Arthritis	0.68
Thyroid Problems	0.09
Heart Problems	0.24
Diabetes	0.73

Salivary HO-1 in non-neurodegenerative and neurodegenerative condition groups

Unadjusted ANOVA results indicated no difference in salivary HO-1 concentration between PD subjects and degenerative neurological controls (*P* = 0.63) and between non-neurological and non-degenerative neurological controls (*P* = 0.26, Table 5). Thus, these groups were combined to make two groups comprising of subjects with neurodegenerative conditions and subjects without neurodegenerative conditions. Table 6 shows the demographic and comorbidity distribution of the newly defined groups. Using these newly defined groups, ANCOVA analysis showed significantly higher salivary mean HO-1 protein level in the neurodegenerative group (64.46 ng/ml; 95% CI: 55.56 – 69.37 ng/ml, n =112) compared to non-neurodegenerative controls (54.39 ng/ml; 95% CI: 48.27 – 60.51 ng/ml, n = 195, *P* = 0.02) after adjusting for age, sex, salivary total protein, and relevant comorbidities (Figure 3, Table 7). Similar to the previous ANCOVA analysis, in this predictor model, total protein was associated to salivary HO-1 (*P* = 0.02) and borderline associations were found between HO-1 and age (*P* = 0.08) and thyroid problems (*P* = 0.09, Table 6).

Table 5. Salivary HO-1 levels

		Unadjusted Analysis		
Group	**n**	**Mean (ng/ml)**	**95% CI**	**P Value**
Non-neurological controls	162	55.79	52.17 - 59.41	0.26*
Non-degenerative neurological controls	33	50.55	42.53 - 58.56	
Degenerative neurological controls	37	65.69	58.12 - 73.26	0.63**
PD subjects	75	63.56	58.24 - 68.88	

*Difference assessed between non-neurological and non-degenerative neurological controls.
** Difference assessed between degenerative neurological controls and PD subjects

TABLE 6 Secondary Analysis: Demographic, comorbidity distribution, and salivary total protein between groups and their relation to salivary HO-1

Groups	**All Non-Degen. Ctl**		**All Neurodegen. Ctl**		***P* Value**
n	**195**		**112**		
Protein (Mean (SD)) (mg/ml)	2.9	1.39	3.79	1.88	0.02
Age (Mean(SD))	62	12	75	10	0.08
Female (%)	99	51%	39	35%	0.61
Male (%)	63	32%	73	65%	
Arthritis (%)	53	27%	42	38%	0.72
No Arthritis (%)	142	73%	70	63%	
Thyroid Problems (%)	39	20%	20	18%	0.08
No-Thyroid Problems (%)	156	80%	92	82%	
Heart Problems (%)	12	6%	21	19%	0.27
No-Heart Problems (%)	183	94%	91	81%	
Diabetes (%)	22	11%	10	9%	0.69
No Diabetes (%)	173	89%	102	91%	

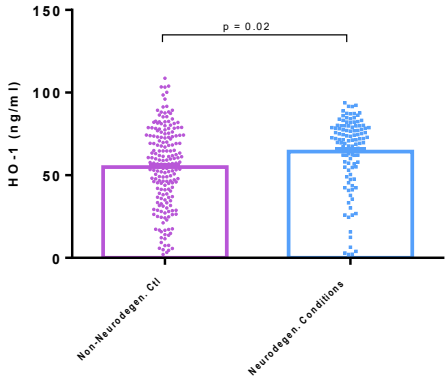

Figure 3. *Adjusted salivary mean HO-1 of patients with neurodegenerative conditions compared to non-neurodegenerative controls.* The study's sample population was redefined into two group and ANCOVA showed elevated adjusted salivary mean HO-1 levels in neurodegenerative group (n = 112) relative to non-neurodegenerative controls (n = 195). Statistical analysis performed using ANCOVA with $\alpha = 0.05$ adjusted for age, sex, salivary total protein, and relevant comorbidities, error bars indicate the mean with SEM.

Table 7. Secondary analysis: Salivary HO-1 levels (adjusted by age, sex, total protein and comorbidities)

		Unadjusted Analysis			Adjusted by Age, Sex, and Comorbidities		
Group	**n**	**Mean (ng/ml)**	**95% CI**	**P Value**	**Mean (ng/ml)**	**95% CI**	**P Value**
Non-neurodegenerative controls	195	54.90	51.61 - 58.20	<0.01	54.39	46.27 - 60.51	0.02
Neurodegenerative conditions	112	64.26	59.92 - 68.61		64.46	55.56 - 69.37	

Logistic regression and ROC curve analyses:

Logistic regression analyses for all comparisons was conducted using salivary HO-1 in the univariate models, as well as HO-1, salivary total protein, age, sex, and relevant comorbidities as predictors in the multivariate models.

Table 8 shows the univariate logistic regression analyses. In these models, salivary HO-1 was associated to PD likelihood in models against non-neurological (OR = 1.01, $P = 0.02$) and non-degenerative controls (OR = 1.03, $P < 0.01$), but not against other degenerative neurological conditions (OR = 1.00, $P = 0.63$).

Table 9 shows the multivariate models. In these models, salivary HO-1 was associated to increased likelihood for PD relative to non-neurological controls (OR = 1.02, $P = 0.01$) and relative to non-degenerative neurological control (OR = 1.04, $P < 0.01$). Salivary total protein was found to be positively associated to PD outcome likelihood relative to non-neurological controls (OR = 1.56, $P < 0.01$). PD was also found to be association with age relative to non-neurological controls (OR = 1.06, $P < 0.01$) and relative to non-degenerative neurological control (OR = 1.08, $P < 0.01$). The inverse association between PD and age was found when compared to degenerative neurological controls (OR = 0.89, $P < 0.01$). PD was more likely to occur in males compared to all controls ($P < 0.01$) with a 6.45 times increased likelihood in the model against non-neurological controls, a 10.45 times increase in the model against degenerative neurological controls, and a 15.35 times increase in the model against non-degenerative neurological controls. PD was also associated with diabetes, with patients with diabetes having lower odds of PD in the model relative to non-neurological control (OR = 0.21, $P = 0.02$) and to degenerative neurological

controls (OR = 0.08, $P < 0.01$). Lastly, PD outcome was associated to thyroid problems, in the model against degenerative neurological controls (OR = 14.31, $P < 0.01$).

Univariate logistic regression models for neurodegenerative conditions relative to non-neurodegenerative control group revealed an association between HO-1 and neurodegenerative conditions (OR = 1.02, $P < 0.01$, Table 8). Multivariate logistic regression showed and association between neurodegenerative conditions and males (OR = 3.27, $P < 0.01$), age (OR = 1.11, $P < 0.01$), and patients with heart problems (OR = 2.57, $P = 0.03$). A positive association was seen between salivary HO-1 (OR = 1.02, 95% CI: 1.01 – 1.03, $P < 0.01$) and total protein (OR = 1.39, $P < 0.01$) with neurodegenerative conditions.

Multivariate logistic regression analyses were used to generate corresponding ROC curves (Figure 4). The area under the ROC curve that separated PD from non-neurological controls was 86% (sensitivity: 0.83, specificity: 0.75), 88% (sensitivity: 0.84, specificity: 0.76) for non-degenerative neurological control and 87% (sensitivity: 0.87, specificity: 0.84) for degenerative neurological control (Figure 4A-C). The area under the ROC curve that separated the neurodegenerative conditions group from non-neurodegenerative controls was 86% (sensitivity: 0.79, specificity: 0.80, Figure 4D).

Table 8: Univariate logistic regression models

	PD v.									Neurodegenerative conditions v.		
	Non-neurological control			Degenerative neurological control			Non-degenerative neurological control			Non-neurodegenerative control		
Predictor	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***
HO-1	1.01	1.00 - 1.03	<0.01	1	0.98 - 1.01	0.63	1.03	1.01 - 1.04	<0.01	1.02	1.01 - 1.03	<0.01

Table 9: Multivariate logistic regression models

	PD v.									Neurodegenerative conditions v.		
	Non-neurological control			Degenerative neurological control			Non-degenerative neurological control			Non-neurodegenerative control		
Predictors	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***	**Odds Ratios**	**95% CI**	***P***
HO-1	1.02	1.00 - 1.03	0.01	1.00	0.97 - 1.02	0.75	1.04	1.01 - 1.07	<0.01	1.02	1.01 - 1.03	<0.01
Total Protein	1.56	1.27 - 1.97	<0.01	1.25	0.94 - 1.70	0.13	1.34	0.97 - 1.93	0.10	1.39	1.15 - 1.68	<0.01
Age	1.06	1.03 - 1.110	<0.01	0.89	0.82 - 0.94	<0.01	1.08	1.03 - 1.15	<0.01	1.11	1.08 - 1.15	<0.01
Sex	6.45	2.94 - 15.24	<0.01	10.45	3.36 - 38.14	<0.01	15.35	4.27 - 69.09	<0.01	3.27	1.75 - 6.25	<0.01
Arthritis	1.15	0.52 - 2.52	0.73	2.91	1.01 - 9.16	0.06	1.00	0.28 - 3.78	1.00	0.87	0.45 - 1.66	0.66
Thyroid problems	2.02	0.77 - 5.38	1.60	14.31	2.91 - 95.60	<0.01	1.75	0.40 - 8.75	0.47	0.87	0.39 - 1.92	0.73
Heart problems	1.89	0.65 - 5.56	0.24	0.99	0.28 - 3.68	0.99	1.19	0.24 - 7.29	0.84	2.57	1.10 - 6.24	0.03
Diabetes	0.21	0.05 - 0.73	0.02	0.08	0.01 - 0.39	<0.01	0.54	0.05 - 5.70	0.61	0.62	0.23 - 1.60	0.33

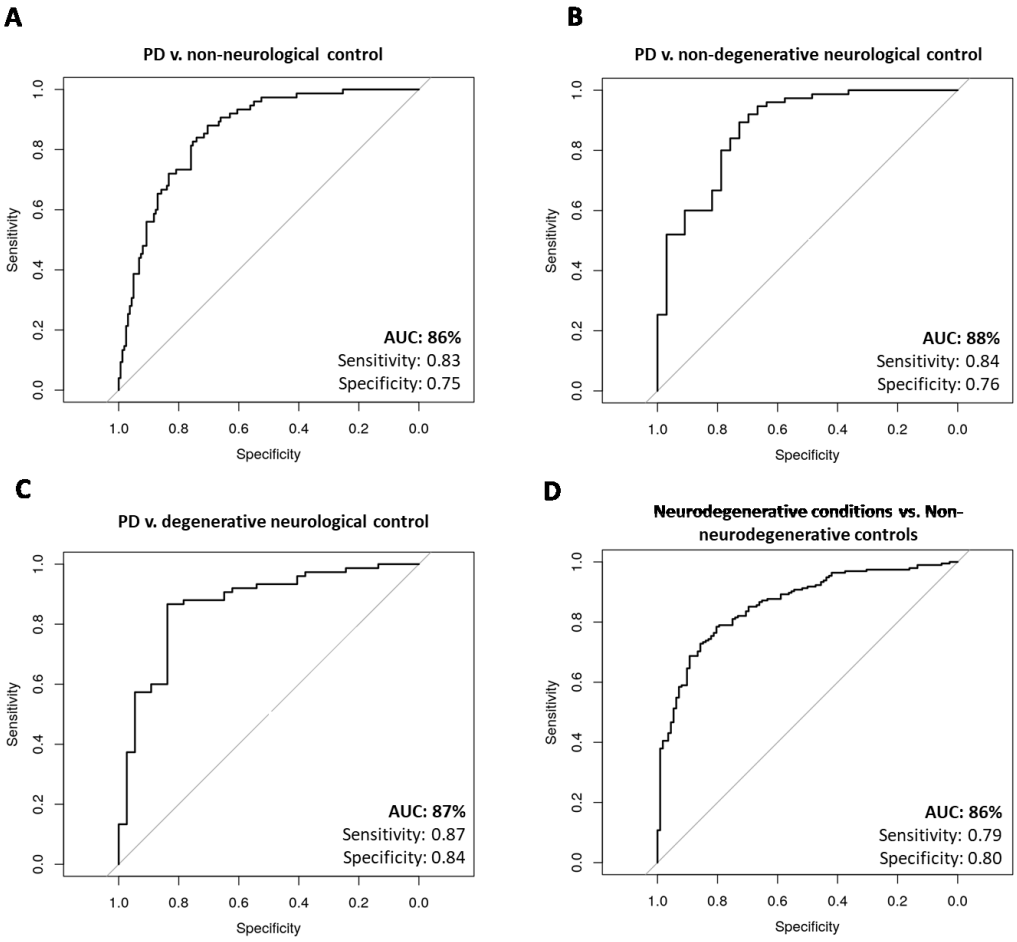

Figure 4. *Receiver operating characteristic curves.* ROC curve with salivary HO-1, salivary total protein, age, sex, and relevant comorbidities as predictors for PD relative to A) non-neurological controls, B) non-degenerative neurological controls, and C) degenerative neurological control, and for D) neurodegenerative conditions relative to non-neurodegenerative controls.

HO-1, disease progression and levodopa equivalent daily dose

A significant difference was found in salivary HO-1 protein levels between non-PD controls (55.97 ng/ml, 95% CI: 50.10 – 61.83 ng/ml, n = 232) and H&Y stage 2 PD subjects (67.04 ng/ml, 95% CI: 57.22 – 77.87, *P* = 0.03, n = 24), but not for later against earlier (H&Y stage 1: 65.14 ng/ml, 95% CI: 53.75 – 76.53, *P* = 0.10, n = 18) or later stage of disease (H&Y stage 3+: 58.05 ng/ml, 95%CI: 47.81 – 68.29, *P* = 0.67, n = 28) with adjustments by age, sex, total protein and relevant comorbidities (Figure 5A). Furthermore, salivary HO-1 was not significantly different among PD patients at HY stage 1, 2 or greater than 3 by ANOVA analysis (*P* = 0.39, Figure 5B).

Medical charts including pro-dopamine medication dosages were found for 70 PD patients and were converted to levodopa equivalent daily dose (LEDD). Mean LEDD (mg) of HY stage 1 PD patients was 441.31 mg (95% CI: 220.76 – 661.86 mg, n = 18), HY stage 2 PD patients mean LEDD was 555.66 mg (95% CI: 348.49 – 762.86 mg, n = 24), and 805.85 mg (613.28 – 998.41 mg, n = 28) for HY stage 3 PD patients. Among the PD patients, mean LEDD (mg) increased with disease progression measure by the H&Y scaling system, with significant increase in medication dosage from HY stage 1 to HY stage 3 patients (*P* < 0.01, Figure 5C). Pearson correlation showed that LEDD was correlated to salivary HO-1 (*r* = -0.38, *P* < 0.01, Figure 5D).

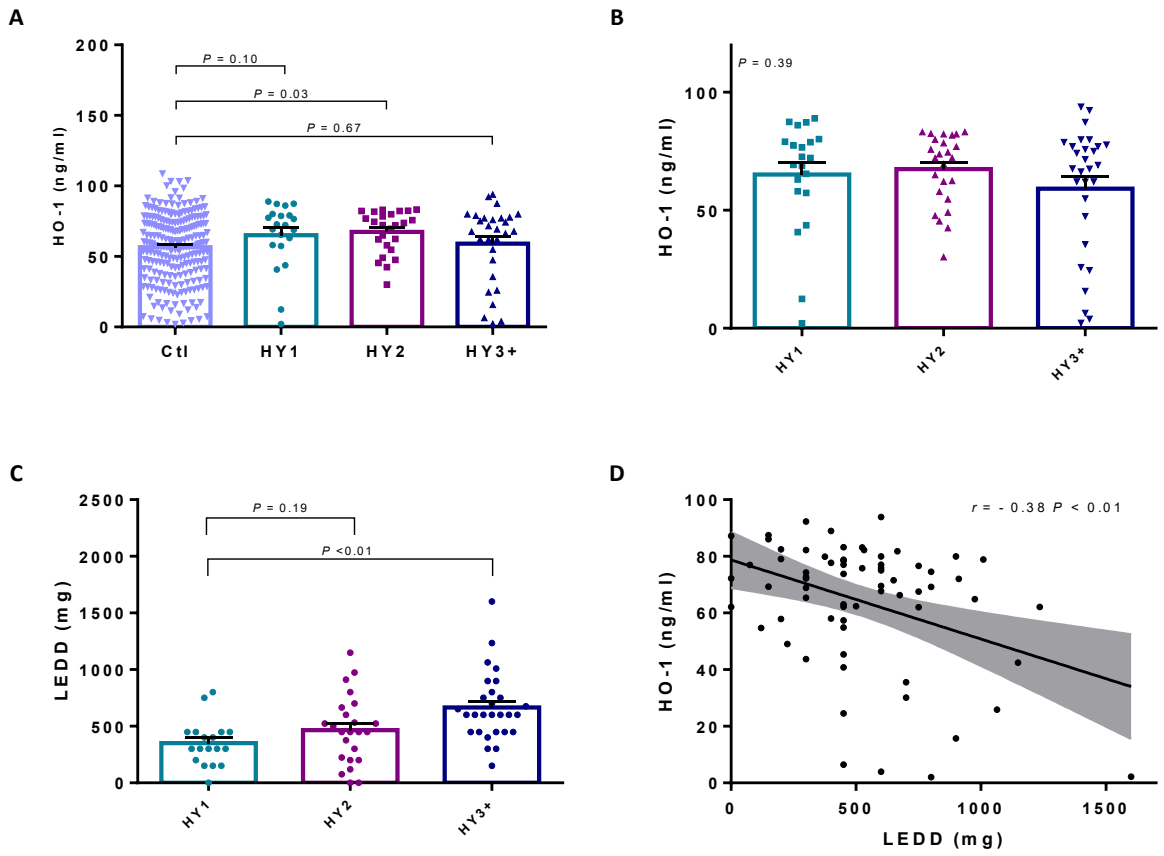

Figure 5. *Relationship between HO-1, LEDD and disease progression and correlation analysis of LEDD with salivary HO-1 protein levels in idiopathic PD patients.* Disease progression measured by H&Y scale progression. A) HO-1 protein levels of all non-PD controls (n = 232) relative to PD cases subdivided into their H&Y stage (HY1 n = 18, HY2 n = 24, HY3 n = 28) and B) HO-1 protein levels and C) LEDD levels among PD patients (n = 70) subdivided into their H&Y stage . Statistical analysis performed using ANCOVA (5A) or ANOVA (5B & 5C) with α = 0.05, error bars indicate the mean with SEM. D) Correlation analysis of LEDD and salivary HO-1 by Pearson correlation.

Discussion:

HO-1 has been implicated in PD pathology, as explored in the review section of this thesis [147, 162, 165-167]. A recent study in 2018, showed for the first time HO-1 protein in saliva, detected by ELISA and western blots of healthy subjects without neurological conditions as well as in PD patients [118]. It was suggested by the authors of the 2018 pilot study that salivary HO-1 had a potential to be a biomarker of PD diagnosis. This pilot study had several limitations including a small sample size, absence of neurological controls as well as no assessment of total protein levels in the saliva to control for potential patient-to-patient variability. In this thesis I aimed to evaluate salivary HO-1 protein levels in a larger sample size comprising of three control groups (non-neurological, non-degenerative neurological and degenerative neurological controls) and PD patients, alongside measuring total salivary protein levels to account for potential variability between patient saliva.

Increased total salivary protein concentration in PD subjects

Measurements of total salivary protein showed an increase in total protein concentration in PD subjects using ANOVA analysis. These results reiterate previous findings by other groups of elevated salivary total protein in PD patients, which was postulated to be caused by PD subjects' autonomic dysfunction [107, 123, 197]. Autonomic disturbances have been suggested to precede motor symptoms. Lewy body pathology, observed through phosphorylated α-synuclein aggregates, were detected in labial and submandibular gland biopsies of idiopathic REM sleep behaviour disorder, a symptom of prodromal PD, and PD subjects and may also perturb normal gland function and salivary content [198, 199]. The symptom of hypersialorrhea

(excessive drooling) observed in PD subjects has been proposed to be in part due to autonomic dysfunction, Lewy body pathology observed in salivary glands and dysphagia, causing an accumulation of saliva rather than increasing saliva production [199, 200]. This is supported by reports of decreased salivary flow rate in PD patients [123, 197, 201]. Both basal and stimulated salivary flow rate in PD patients during the off (absence of levodopa, prior to taking levodopa) state and on (with levodopa administration) state have been reported to be decreased, alongside increase in amylase secretion, a highly abundant protein in saliva, and total protein concentration [123, 197]. This lends to credibility of the idea that the hypersiallorhea in PD patients is due to dysphagia as there is reduced salivary flow rate in these patients. This resulting reduction in saliva production may lead to increased salivary protein concentrations as seen with increased amylase levels and reports of increased total protein concentrations [107, 123, 197].

Furthermore, ANCOVA analysis showed that the salivary total protein as a covariate significantly adjusts HO-1, indicating a potential relationship. This is contrary to unadjusted salivary HO-1 not correlating with salivary total protein, suggesting that the HO-1 protein level is not solely dependent on altered total protein concentration. This may indicate that the altered levels of HO-1 are not solely dependent on altered total protein concentrations but that there still may be an underlying unknown relationship.

Salivary HO-1 protein

Salivary HO-1 was shown to be significantly elevated in PD patients with respect to non-neurological and non-neurodegenerative controls before and after adjustments for potential covariates such as age, sex, total protein, and relevant comorbidities. Salivary HO-1 in

combination with the covariates can correctly classify a person to the PD population apart from the non-neurological and non-degenerative neurological control populations 86% and 88% of the time, respectively. Despite the lack of significant difference in salivary HO-1 levels between PD and degenerative neurological controls, we were still able to differentiate the PD population 87% of the time through the use of a combination of predictors such as age, sex, and relevant comorbidity data used in the previous analysis. These results suggested that a model using HO-1 in combination with the other covariates as predictors may be used as a potential biomarker signature to distinguish the PD population. Although univariate logistic regression showed an association between HO-1 and PD in several models, the use of salivary HO-1 alone was not sufficient to differentiate PD better compared to models with the combination of other covariates as predictors (data not shown). Thus, the use of HO-1 as a biomarker for PD in combination with other potential biomarkers or risk factors of the disease may be a promising avenue of research for the development of a diagnostic test of PD.

Another promising avenue of biomarker research in PD is the investigation of potential biomarkers in salivary extracellular vesicles. Reports have shown the presence of potential PD biomarkers in extracellular vesicles of various biofluids [109, 114, 117]. A promising potential biomarker of PD, salivary oligomeric α-synuclein, was reported to have a diagnostic accuracy of 72.4% (sensitivity 76%, specificity 60%) relative to non-PD healthy controls when using whole unstimulated saliva [115]. The diagnostic accuracy of oligomeric α-synuclein as a marker of PD was 94.1% (sensitivity 92%, specificity 86%) relative to non-PD healthy controls in isolated extracellular vesicles from whole saliva [114]. This increase in accuracy of over 20% by assessing a potential biomarker in extracellular vesicles has heavy implication for future biomarker

research, if it can be recapitulated in other biomarker studies. Furthermore, a recent study reported that HO-1 was mainly localized in extracellular vesicles of various biofluids including saliva, plasma, serum, urine and cerebrospinal fluid [202]. Furthermore, it was reported that a substantial proportion HO-1 localized in extracellular vesicles were derived from the central nervous system, specifically in L1 cell adhesion molecule-enriched and glutamate aspartate transported-enriched extracellular vesicles fractions [202]. Based on these recent findings, investigating HO-1 levels in isolated extracellular vesicles from saliva may yield promising results for biomarker research in PD. In addition, if many potential biomarkers can be found in these vesicles, further isolation of extracellular vesicles from certain cell population groups such as neuronal or glial cells can be insightful in future research.

Effects of Levodopa medication:

Salivary HO-1 was found to not be significantly different among PD patients at different stages of disease progression based on the H&Y scaling system. However, HO-1 protein levels were higher in HY stage 1 (65.14 ng/ml), HY stage 2 (67.04 ng/ml), and HY stage 3 or greater (58.05 ng/ml) compared to non-PD controls, with significant difference seen between non-PD controls and PD HY stage 2 patients, and a trend increase in HY stage 1 patients. These results may be due to the association found between salivary HO-1 and LEDD. The increase in salivary HO-1, particularly at HY stage 1 and 2, seen in this study in PD subjects may be reflective of chronic HO-1 expression as seen in animal models and PD patients.

There have been numerous papers indicating the negative effects of chronic expression of HO-1 in PD pathology in both humans and animal models [147, 162, 165-167]. In particular,

chronic expression of human HO-1 in a transgenic mice model during mid-to-late life led to these animals to express PD-like phenotypes including motor incoordination [162, 167]. Chronic oxidative stress as seen by HO-1 immunohistochemistry in PD post-mortem brain specimens were also observed in other studies [147, 166]. While PD HY stage 3 patients, still showed higher HO-1 than non-PD subjects, the increased levels were not as high compared to PD patients at earlier stages of disease. This may be due to the negative association between HO-1 and LEDD seen among PD patients in this study. LEDD was also seen to be higher with disease progression (measured by the HY scaling system), and higher dosage was likely prescribed to alleviate worsening of symptoms. The high dosage of levodopa among HY stage 3 or greater patients may have been sufficient in reducing HO-1 protein levels in saliva relative to subjects at earlier stages of PD. This may explain the lack of significant results of HO-1 levels being different with disease progression among the PD patients.

Elevated salivary HO-1 in neurodegenerative conditions

While salivary HO-1 in combination with other covariates was able to differentiate the PD population from the control groups in this study, the lack of statistically significant difference between the PD and degenerative neurological control groups, brings into question whether salivary HO-1 is a biomarker specific for PD or may better serve as a neurodegenerative condition biomarker in spite of the ROC curve results. To reflect previous results, the study's sample population was redefined into a neurodegenerative condition group and a non-neurodegenerative control group was done to investigate this question. This secondary analysis revealed that salivary HO-1 is significantly elevated in neurodegenerative conditions such as PD, AD, and MCI, compared to subjects without neurodegenerative conditions and able to distinguish

the two groups apart with an AUC of 84.3%. While this is lower accuracy than the models differentiating the PD population to non-neurological and non-neurodegenerative groups, it gives a better foundation in future research into potential diagnostic biomarkers for PD.

Effects of covariates on HO-1:

A borderline association was found between salivary HO-1 and age in ANVOCA. This result is in line with a previous study on salivary HO-1 in PD and non-neurological controls showing an association between age and salivary HO-1 protein level [118]. An association with age and HO-1 may be linked to the oxidative damage and mitochondrial damage of aging. In these theories, aging is partly caused by increased oxidative stress and damage to cells. Oxidative damage and mitochondrial damage have been linked to altered life expectancy in animal models and humans, supporting these oxidative stress and damages it can cause is a factor in normal aging [131-135]. Under basal conditions in the adult mammalian brain and other tissues, apart from the spleen and gastrointestinal tract tissue, HO-1 expression is low. HO-1 can be induced by a variety of oxidative stress stimuli and as a marker of oxidative stress alterations in HO-1 expression induced by oxidative stress may be linked with age [148-150]. As age has been shown to be linked with oxidative damage and mitochondrial damage, research into the potential relationship between HO-1, a marker of oxidative stress, and aging have been conducted although information is still limited [131, 203-207].

Although information linking HO-1 with age is limited, it has been reported that there is a progressive increase in HO-1 immunoreactivity in neuroglia of the human brain in an age-dependent manner [203]. Additionally, an age-dependent increase in HO-1 protein expression

was seen in the mouse cortex, and increased HO-1 mRNA expression at the choroid plexus also increased with normal aging in rats [204, 205]. However, other studies reported the inverse relationship of HO-1 with age, and an age-related decrease in HO-1 protein and mRNA observed in the hippocampus and substantia nigra brain regions and specifically in hippocampal astrocytes in aged rats [206, 207]. Despite the limited literature on the topic, altered HO-1 expression, a marker of oxidative stress, has been linked to normal aging.

A borderline association between salivary HO-1 and thyroid problems was also found during ANCOVA analysis. It has been reported that HO-1 expression can be induced by thyroid hormone 3,5,3'-triiodothyronine (T3) and *L*-thyroxine (T4) administration in rat liver and in a thyroidectomized rat (hypothyroidism) model [208-210]. The majority of subjects with thyroid problems in this study was composed of those by hypothyroidism and were taking Synthroid a T4 hormone replacement for their condition. It is possible that this borderline association with HO-1 and those with thyroid problems can be caused by thyroid hormone-induced HO-1.

Predictors of PD or neurodegenerative conditions:

As previously mentioned, despite salivary HO-1 levels not differing between the PD group and those with other neurodegenerative conditions, multivariable logistic regression results showed that salivary HO-1 in combination with other covariates as predictors was able to differentiate the PD group from the controls. This was due to the combination of covariates used as predictors of PD, specifically age, sex, thyroid problems, and diabetes.

Increased salivary HO-1 and total protein was associated with the likelihood of PD in several of the models presented here. Furthermore, multivariable logistic regression showed an

association of age, sex, thyroid problems, or diabetes to the likelihood of having PD. In all PD models, the likelihood of having PD increased with age and in males. These results are in line with other literature indicating that increasing age is a major risk factor for PD and PD prevalence in males being 1.40 times higher than females [1, 23-25]. In addition, diabetes mellitus is reported here to be inversely associated with PD outcome in several models. This inverse association between PD and diabetes have been reported by various groups [76, 78]. However, other groups have also reported that diabetes, in particular Type 2 diabetes, was associated with increased risk of PD as well as others have reported no association between the two [72, 73, 75, 77, 79].

Thyroid problems were also found to be associated with increased likelihood of having PD by 14.31 times compared to those with other degenerative neurological conditions such as AD and MCI. This finding of a positive association between PD and thyroid problems is in line with a previous report [70]. The majority of patients included in this study who indicated that they experience thyroid problems had hypothyroidism or is taking Synthroid, a medication to provide T4 hormone for low thyroid hormone therapy. Patients with hypothyroidism has been previously reported to a nearly 2 times increased risk of developing PD in a Taiwan nation-wide study consisting of 4725 cases and matched controls [70]. Hyperthyroidism has also been associated to PD and exacerbated parkinsonian motor complication [211, 212]. Thyroid hormone levels, such as lower TSH and free T3, have also been associated to motor symptoms in PD subjects [213], further linking thyroid problems with PD.

Increasing salivary HO-1 and total protein levels in saliva by 1 unit was also shown to increase the likelihood of having a neurodegenerative condition such as PD, AD, and MCI. Increasing age and sex were not surprisingly associated with increased likelihood of having

neurodegenerative diseases. The worldwide prevalence of these conditions is known to increase with age [1, 214]. In addition, neural degeneration has been seen in normal aging at a rate of 4.7% per decade in the substantia nigra pars compacta with exponential increase in neurodegeneration in human PD brain specimens with a 45% loss within the first decade [8]. This once again may tie into the oxidative damage and mitochondrial damage theory of aging, as in these patients and models of these diseases do exhibit signs of oxidative stress and mitochondrial damage with age. The association of sex to neurodegenerative diseases is also not surprising as there is a sex bias in both PD, AD and other dementias with the prevalence of PD have been shown to be 1.40 times higher in males than females while there is a 1.17 times female predominance in Alzheimer's disease [1, 214].

Lastly, heart problems were found to be associated with decreased likelihood of having a neurodegenerative condition relative to non-neurodegenerative controls. ROS-mediated endothelial dysfunction leading to cardiovascular tissue injury is a pathological factor for cardiovascular disease (CVD) [215]. The neurodegenerative conditions include in this study, particularly AD and PD, have links to several risk factors for CVD. Hypertension was reported to accelerate tau-related pathology as well as has been shown as a risk factor for CVD [216]. Other risk factors for CVD such as hypercholesterolemia, hyperhomocysteinemia, and oxidative stress was also been shown to be linked to AD pathology [217]. Additionally, the prevalence for cardiac failure was two times higher in the PD population relative to the normal population and was associated with elderly onset of PD [218].

While there are many potential risk factors for neurodegenerative diseases and potential covariates influencing HO-1 expression, the results in this thesis show, i) elevated levels of

salivary HO-1 in PD patients compared to subjects without neurodegenerative conditions, ii) elevated levels of salivary HO-1 protein in patients with neurodegenerative conditions, iii) salivary total protein levels are higher in PD subjects but is not a sole factor to the elevated levels of HO-1, iv) an association between PD and salivary HO-1, total protein, age, sex, diabetes and thyroid problems, and v) an association between neurodegenerative diseases and age, sex, and heart problems. This paper also shows that HO-1 in combination with the covariates included in this study, has the potential to differentiate those with neurodegenerative conditions, and with further research and in combination with other biomarkers more specific to PD may be used to differentiate the PD population.

Limitations

There were several limitations to the current study presented. Firstly, the sample population included were recruited from a single site and a replication cohort is lacking. Secondly, the sample size for the degenerative and non-degenerative neurological control group was smaller than the others. Thirdly, this study does not include PD-mimics such as patients with progressive supranuclear palsy and multiple system atrophy. Lastly, MDS-UPDRS scores were not available to corroborate H&Y staging for measurement of disease progression and severity and should be included in future studies for PD staging. Addressing these four limitations to this study would make future studies more robust.

Conclusion:

Validation of a quantifiable salivary biomarker of idiopathic PD in a large-scale trial would address an unmet clinical need for rapid and accurate diagnosis of this condition. The use of salivary HO-1 alongside demographic and comorbidity data in a predictor model proved to be sufficient in differentiating the PD population and patients with neurodegenerative conditions. The inclusion of another potential biomarker more specific for PD such as salivary oligomeric alpha-synuclein as well as measurements of biomarker in the extracellular vesicles in the biofluid may provide a more accurate model to distinguish the PD population. Future studies of salivary HO-1 as a potential biomarker of disease should investigate these two avenues.

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