Review Article

Salivary biomarkers for the diagnosis and monitoring of neurological diseases

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ABSTRACT

Current research efforts on neurological diseases are focused on identifying novel disease biomarkers to aid in diagnosis, provide accurate prognostic information and monitor disease progression. With advances in detection and quantification methods in genomics, proteomics and metabolomics, saliva has emerged as a good source of samples for detection of disease biomarkers. Obtaining a sample of saliva offers multiple advantages over the currently tested biological fluids as it is a non-invasive, painless and simple procedure that does not require expert training or harbour undesirable side effects for the patients. Here, we review the existing literature on salivary biomarkers and examine their validity in diagnosing and monitoring neurodegenerative and neuropsychiatric disorders such as autism and Alzheimer's, Parkinson's and Huntington's disease. Based on the available research, amyloid beta peptide, tau protein, lactoferrin, alpha-synuclein, DJ-1 protein, chromogranin A, huntingtin protein, DNA methylation disruptions, and micro-RNA profiles display a reliable degree of consistency and validity as disease biomarkers.

Neurological disorders are a family of diseases affecting both the central and peripheral nervous system, ranging from neurodegenerative to neurodevelopmental and psychiatric. Many of these diseases are multifaceted and there is no consensus about their actual cause, although many studies suggest the involvement of multiple combined factors [1,2]. One aspect of neurological disorders is neurodegeneration, which is caused by a progressive loss of certain classes of neurons that affect either motor skill or memory and cognition. The degeneration of these neurons is usually due to

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several molecular mechanisms that promote cell death including excitotoxicity, mitochondrial dysfunction, and intracellular inclusions or extracellular aggregation of toxic molecules [3]. Early disease detection is critical in assigning proper treatment therapy to affected patients [4]. Yet diagnosis remains a challenge concerning diseases affecting the central nervous system (CNS) due to multiple delays in the diagnostic procedure and treatment initiation, which ultimately diminishes treatment effectiveness. Usually the tests performed for the diagnosis of neurological conditions are either blood tests or lumbar puncture. Their invasive nature, especially for the lumbar puncture, usually results in discomfort, pain and disagreeable side effects for patients, which necessitates the search for accurate, more advanced and less invasive testing methods [5]. Recent investigations have begun to examine the possible use of urine as a less invasive source of biomarkers, but have not yet been put into clinical use pending further assessment [6–9].

Saliva is a physiological fluid composed of mucous and serous secretions containing mucin, alpha-amylase and different ions [10]. It fulfils a range of functions including: digestion of nutrients and protection of teeth and oral tissues; through enzymatic action, lubrication and antibacterial properties [11]. Saliva is secreted in the mouth by the principal salivary glands: the sublingual, submandibular and the parotid, which are under direct parasympathetic innervation of the cranial nerves VII (glossopharyngeal) and IX (facial). The facial nerve innervates both the sublingual and submandibular glands through the submandibular ganglion, while the parotid gland is under glossopharyngeal innervation via the otic ganglion [12]. Therefore this close relation between the salivary glands and the nervous system could render these glands' secretions as a useful pool of biomarkers that represent various normal and pathological physiologies of the nervous system [13]. Saliva offers a new and easily accessible physiological fluid that can be collected in a non-invasive manner and assessed using different analytical assays [14]. Although many diseases have confirmed salivary biomarkers [15]; diseases affecting the nervous system have few markers

available in saliva which are still being investigated. This narrative review is concerned with the salivary biomarkers of the most known neurological diseases i.e. Alzheimer's, Parkinson's Huntington's, Amyotrophic lateral sclerosis, Multiple sclerosis, Autism spectrum disorders and finally neuropsychiatric disorders, to establish a general idea about the advances made in this field in hopes of providing guidelines for the development of methods to monitor and assess nervous system health using salivary biomarkers.

Alzheimer's disease

Alzheimer's disease (AD) is a chronic neurodegenerative disorder of the CNS. It is mainly manifested by dementia, confusion and cognitive impairment due to the loss of neurons in the hippocampus, basal forebrain and other cortical areas of the brain [16–18]. AD etiology can be attributed to two molecules, the amyloid beta peptide and tau protein. Amyloid beta peptide is proposed to have normal physiological roles in memory formation, lipid homeostasis, regulation of neuron activity and neurite growth [19–24]; on the other hand, tau protein is a member of the microtubule associated proteins that maintain proper neuronal structure and intracellular transport [25–27]. Studies on AD pathophysiology suggest that extracellular accumulation of amyloid beta peptides (Aβ) in amyloid plaques and intracellular tau protein neurofibrillary tangles are the major factors that contribute to neuron cell death [28–33]. Based on different research efforts, the cause of neurodegeneration cannot be attributed to only one of the aforementioned key players in disease pathophysiology rather than a combinatory effect of both amyloid beta and tau protein pathologies on neuronal cell death.

Amyloid beta peptides

Using animal models and neural cell lines, functions of the soluble APP alpha isoform have been described which encompass neurogenesis, neurite outgrowth, neurite

Table 1 Normal physiological and neurotoxic functions of the derivatives of APP processing. The following table lists the functions that are known to be performed by the derivatives of APP processing. It is worth noting that sAPPβ and p3 are missing due to lack of information regarding the function of these two derivatives.

sAPPα	Aβ		AICD
Normal physiological function	Normal physiological function	Neurotoxicity	Neurotoxicity
- Cell survival - Synaptogenesis - Neurogenesis - Neurite outgrowth - Learning and memory - Long term potentiation - Long term depression - Suppress Aβ generation - Modulate β-secretase activity - Regulation of glutamate receptors [34,35,325]	- Lipid homeostasis - Cholesterol regulation - Lipoprotein anti-oxidant - Transcription factor - Neurite outgrowth - Neural viability - Memory formation - Regulation of synaptic activity - Neurogenesis [19–23,326,327]	- Plaque generation - Neurite damage - Oxidative stress - Calcium influx - Pro-inflammatory - Synaptic dysfunction - Apoptosis - Inhibition of long term potentiation [328–333]	- Transcription factor - Pro-apoptotic - Inactivation and degradation of Aβ - Trigger of apoptosis - Cytoskeletal destabilization - Mitochondrial dysfunction - Pro-inflammatory - Increase intracellular calcium - Up regulation of APP and BACE [334,335]

guidance, axonal transport, learning, memory formation and synaptogenesis [34,35] [Table 1]. In non-pathologic conditions, amyloid precursor protein is cleaved first by α-secretase a member of the ADAM family followed by a subsequent cleavage by γ-secretase. These two proteases are involved in the normal processing and recycling of transmembrane amyloid precursor protein. Normally, APP processing by α-secretase cuts within the amyloid beta sequence and results in the formation of three main peptides: soluble APP alpha (s-APPα), which is released into the extracellular space, and an 83 AA fragment (C-83) embedded in the membrane. C83 is finally cleaved by γ-secretase releasing the p3 peptide and the amyloid precursor protein intracellular domain (AICD) [36,37] [Table 1] [Fig. 1].

In AD, α-secretase is substituted by β-secretase known as BACE-1 which does not cleave APP within the beta amyloid structure, thus maintaining its integrity and function. This altered sequence in enzymatic cleavage results in the production of (i) a soluble amyloid precursor protein beta (s-APPβ), and (ii) a 99 AA fragment (C-99) that is subsequently cleaved by γ-secretase to produce a 38–43 amino acid residue amyloid-β peptide and the intracellular AICD domain [38,39]. The amyloid beta 42 (Aβ42) isoform which is known to be more amyloidogenic and neurotoxic than the other isoforms, accumulates into plaques causing adverse cytopathic effects [40–42]. This cleavage can also produce the Aβ40 isoform, which has been found to enhance and stabilize Aβ42 oligomer. The synergistic
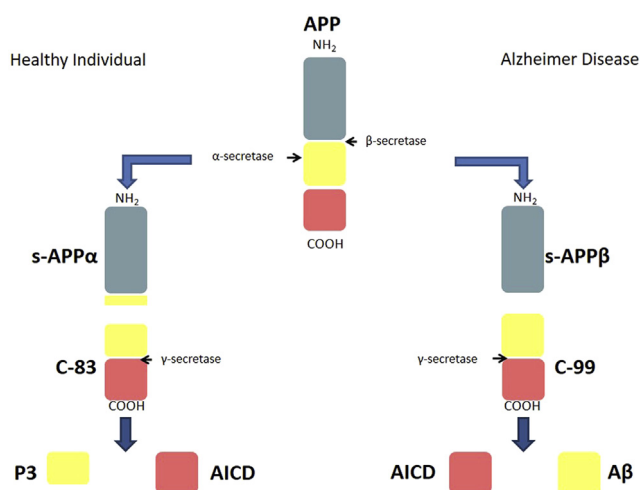
Fig. 1 **The normal and AD pathogenic pathway of APP processing into its multiple derivatives**. In a healthy individual the primary processing of APP by α-secretase within the Aβ sequence produces the s-APPα which is released to the extracellular domain. This cleavage of the Aβ domain inactivates its amyloidogenic potential. The cleavage of the produced C-83 peptide by γ-secretase produces the P3 peptide which is released to the extracellular space and the AICD which remains inside the cell. The difference between the normal APP processing pathway and that of AD is the initial cleavage of APP by β-secretase in AD, producing an s-APPβ and conserving the Aβ sequence. Aβ is then released after C-99 processing by γ-secretase. Once secreted to the extracellular space, Aβ is free to aggregate and form amyloid plaques.

effects of the two Aβ isoforms instigate severe neurite damage along with neuronal toxicity [43–46] [Fig. 1] [Table 1].

Applying the enzyme-linked immunosorbent assay (ELISA) to saliva samples, Bermejo Pareja et al. [47] compared amyloid beta 40 and 42 levels between AD patients and two sets of controls: healthy controls and Parkinson patients. It was evident that Aβ42 levels increased in patients suffering from mild and moderate AD, but Aβ42 levels were comparable to the healthy controls in severe AD. This reported variation in Aβ42 concentrations with disease progression, mimics previously established CSF findings [48–50]. Aβ40 levels remained unchanged using this technique for all participants. A single study reported the detection of Aβ42 as a urinary biomarker for both AD diagnosis and monitoring, with patients suffering from severe dementia exhibiting a complete absence of Aβ42 [9].

In contrast to these findings, Kim et al. [51] used antibody-based magnetic nanoparticle immunoassay to show that Aβ42 secretion increases with disease progression from mild cognitive impairment to severe AD; an increase not evidenced in CSF, but in the brain as deposition of intracerebral Aβ42 in amyloid plaques [49]. Aβ40 levels also exhibited an increasing trend similar to that of Aβ42 as opposed to being unchanged in the previous study but remained without statistical significance. Kim et al. have also conducted an ELISA on the saliva samples and the results were in accordance with their findings using the immunoassay. There exist two scenarios that might explain the variability of Aβ42 concentration between the two studies. The first being that, excessive loss of intracerebral neurons in advanced AD contributes to the decrease of released Aβ42 which explains the results obtained by Bermejo-Pareja et al. [47] The second being that, AD induced reduction in submandibular salivary flow might contribute to the increase of concentration of Aβ42 due to decreased sample volume which explains the results obtained by Kim et al. [47,51]. Yet there is still no consensus or precise explanation for the observed contradiction.

Recent work performed by Lee et al. [52] proved that stabilizing salivary levels of Aβ42 peptides by adding thioflavin S to prevent its aggregation, and inhibiting bacterial growth by using sodium azide greatly enhanced the results obtained by ELISA. These compounds act as preservatives and prevent sample degradation and thus enhance sample quality and Aβ42 detection. Aβ42 detection by using this method accurately differentiated between controls and individuals at risk or affected by AD. The concentration of Aβ42 in healthy controls was around 20 pg/ml and in individuals affected by or at risk of developing AD was above 40 pg/ml. Lee et al. did not report any differences in Aβ42 concentrations for different disease stages (mild-moderate-severe) nor did they attempt the detection of Aβ40 with their method. To test the efficiency of the methods used by Bermejo Pareja et al., Lee et al. performed an ELISA using the same Invitrogen kit used by Bermejo Pareja et al. and found that it was only capable of detecting 25% of Aβ42 in the sample when compared to their method.

Using Parkinson patients as controls is essential to prove the validity and exclusive nature of this diagnostic tool in identifying amyloid beta exclusively in AD patients. Results had shown that controls suffering from Parkinson's disease studied by both Bermejo Pareja et al. and Lee et al. showed no differences in Aβ42 concentrations in comparison to healthy

controls and thus proving the specificity of salivary Aβ42 as a salivary biomarker for AD patients. This comes as sound evidence with the findings of amyloid beta pathology in PD patients [53–55].

Molecular findings have shown that multiple genes have been identified as key players in AD diagnosis and prognosis. The four main genetic mutations in AD target PSEN1, PSEN2, ADAM10 and APOE. PSEN1 and PSEN2 are both subunits of the γ-secretase complex and have been associated with the familial cases of AD [56]. ADAM10 is an α-secretase and APOE4 is a member of the apolipoprotein family which plays a role in lipid metabolism and is speculated to intervene in amyloid beta plaque formation. Both ADAM 10 and APOE4 mutations have been associated with late onset AD [57–59].

Lee et al. [52] reported three non-AD subjects who showed Aβ42 concentrations higher than other controls two of whom had an extensive family history of AD and one with a PSEN1 mutation. Complimentary findings were provided by Bermejo Pareja et al. [47] who reported that Aβ42 concentrations in the saliva of AD are independent of APOE4 genotype which is linked to late onset AD. Given the above presented data about the implication of PSEN1 mutations in familial AD, it could be concluded that salivary Aβ42 is probably more reflective of familial AD genotype rather than sporadic AD.

Aβ40 concentrations did not show an identical uniform variation between the two stated studies. Aβ40 levels showed no change between the saliva of patients and of controls with the ELISA as shown by Lee et al. [52] and Bermejo-Pareja et al. [47]. On the other hand Aβ40 levels followed an increasing pattern in AD patient saliva that did not reach significance as demonstrated by Kim et al. [51] using the immunoassay.

Tau protein

Tau protein, known as the Microtubule Associated Protein T (MAPT), is a member of the microtubule associated protein family which plays a role in microtubule stabilization and flexibility by binding to tubulin [26,27]. Normally, tau phosphorylation promotes its disassembly from microtubules and initiates its destabilization and elimination [25,60]. Mutations in the tau protein sequence altering its phosphorylation site have been reported to induce tau hyper phosphorylation [61]. Phosphorylated tau (p-tau) can aggregate and form intracellular neurofibrillary tangles and work synergistically with Aβ to enhance its cytotoxic functions [28,61,62]. Following Mass Spectrometry analysis of patient saliva, Min Shi et al. [13] were the only group who examined p-tau as a salivary biomarker for AD. Analysis of tau protein species, which are the phosphorylated tau (p-tau) and the total tau (t-tau), conveyed that the ratio p-tau/t-tau per individual exhibited a significant increase in affected individuals as compared to healthy controls. This indicates that the increase of phosphorylated tau with respect to the total tau concentration could be used as a potential biomarker for assessment of AD status. It is worth noting that both CSF p-tau and t-tau exhibit an increase in AD patients [63].

Acetylcholinesterase activity

Acetylcholinesterase inhibitors (AChE-I) are the primary medications for AD symptom management and disease control

[64,65]. Acetylcholinesterase is the enzyme required to degrade Acetylcholine (AChE) neurotransmitter released into the synaptic cleft to halt its postsynaptic effects. The Nucleus Basalis of Meynert is a major cholinergic nucleus of the basal forebrain which is a principal target of neurodegeneration in AD [66]. AChE has been demonstrated to accumulate in amyloid plaques and neurofibrillary tangles of AD brains; furthermore many findings have implicated AChE in Aβ pathology and enhancing neurotoxicity [67–69]. Assessment of AChE catalytic activity in AD patients by conventional methods such as positron emission tomography showed a significant decrease in this activity in the affected brain regions [70,71]. Sayer et al. [72] were the first to report AChE salivary activity by using the Ellman colorimetric method. They reported that, AChE activity decreases with age among healthy individuals, and was significantly decreased in patients with AD who did not respond to AChE-I treatment. While those who respond to AChE-I treatment did not show any variation when compared to controls.

Further studies conducted by Bakhtiari et al. [73] and Boston et al. [74] also using the Ellman colorimetric method, found a decreasing trend in AChE catalytic activity in AD patients as compared to controls but these results did not reach significance. They both attributed the decrease in catalytic activity observed by Sayer et al. to long term adaptive changes in the production of AChE because of the administered AD medication.

Lactoferrin

Current research continues to supply evidence implicating the immune system as a major player in the course of AD [75,76]. Recent work presented by Carro et al. names lactoferrin as an impressive new candidate to be one of the first salivary biomarkers for AD early detection and diagnosis [77]. Lactoferrin is an antimicrobial peptide which targets bacteria, viruses, fungi, yeasts and protozoa with a known Aβ-binding ability. It functions in the modulation of immune reactions and inflammation [78–87]. The fact that there exists concomitant evidence implicating systemic and brain infections with AD [88–91], further emphasizes the validity of lactoferrin as a probable biomarker for AD. Using Mass spectrometry and ELISA, Carro et al. [77] demonstrated that salivary lactoferrin concentration was significantly reduced in AD patients when compared to healthy controls, and controls suffering from PD. They were also able to prove the value of lactoferrin in early disease detection as an early disease biomarker. 14 out of 18 controls who presented with reduced salivary lactoferrin concentrations comparable to AD associated concentrations developed either mild cognitive impairment or AD over the course of the study; while, none of the controls who presented with normal or high lactoferrin salivary concentrations developed any form of cognitive impairment. Finally, to establish the validity of this marker and prove that it is truly representative of AD pathology, significant correlations were established between lactoferrin levels in saliva and APOE4 allele status, Mini Mental State Examination (MMSE) score, CSF Aβ$_{42}$ and CSF total tau.

Protein carbonyl levels

There is much evidence concerning oxidative stress being implicated in AD, with reports indicating a wide array of

processes involved in, or resulting from free radicals, such as lipid peroxidation, DNA, RNA and protein oxidation [92]. Protein carbonyls originate from protein oxidation. In the process of carbonylation of proteins, carbonyl side chains are added to proteins as they undergo oxidation reactions [93]. Protein carbonyls were found to be elevated in multiple brain regions in AD subjects such as the hippocampus, parahippocampal gyrus, inferior parietal lobule, superior and middle temporal gyri [94,95].

Haixiang Su et al. [96] were able to quantify protein carbonyl levels by ELISA in saliva samples of AD patients and found that there is no significant difference between AD and controls, though they were able to identify a diurnal variation in carbonyl levels that peak at 2 pm. This intriguing peak was maintained in both AD patients and control, with dampened level in APOE 4 genotype patients but this dampening remained non-significant.

Parkinson's disease

Parkinson disease (PD) is a progressive neurodegenerative disorder resulting in multiple motor and cognitive deficits. The symptoms of this disorder have been classified into two groups, motor and non-motor [97] [98]. In most cases the specific cause of PD remains unknown and the disease has been termed idiopathic; although, familial forms and environmental risk factors have been identified [99,100]. PD mainly causes loss of two classes of neurons across the brain: the dopaminergic and the serotoninergic. This neuron loss triggered by the aggregation of alpha-synuclein proteins within neurons in structures known as Lewy bodies, the hallmark of PD, leads to the multiple motor and non-motor deficits associated with Parkinson [101–103]. PD is classified into three main types based on the dominant symptom: tremor dominant type, akinetic-rigid dominant type and the mixed type [104].

Alpha-synuclein

Alpha-synuclein (α-syn) is a 140 amino acid protein and member of the synuclein family. It is abundant and ubiquitously found in multiple brain regions such as the striatum, hippocampus, olfactory bulb, neocortex, thalamus and cerebellum [105]. It is located in the presynaptic terminals of neurons and has shown affinity for SNARE complex proteins such as synaptobrevin-2, synapsin III and rab3A. α-syn has been found to affect synapsin III expression and its cellular distribution in dopaminergic neurons, the caudate and putamen of PD patients [106–109]. This results in shifts in neurotransmitter vesicle cluster arrangement and dopamine release. Alpha-synuclein is known to exist in and cycle between two forms: (i) the soluble cytosolic form which has no known function [110], and (ii) the membrane bound helical form which functions in membrane fusion in the cascade of synaptic events [111]. Alpha-synuclein pathology in familial PD has been linked to its increased concentrations in neurons, which can be attributed to increased α-syn gene (SNCA) expression due to promoter polymorphisms as well as gene copy number duplication and triplication [112–115]. Along

with gene overexpression, three point mutations in the SNCA gene sequence have been identified in familial PD that affect α-syn aggregation [116–121]. There are two forms of α-syn aggregation in PD: (i) oligomeric amorphous aggregates which are linked to multiple organelle dysfunctions as well as defects in the axonal transport system [122–130], and (ii) fibrillar insoluble aggregates that contribute to the formation of Lewy Body pathology [131–133].

In the quest to establish α-syn as a prominent salivary biomarker of PD diagnosis, Devic et al. [134] used western blotting and spectrometry to establish the presence of α-syn in human saliva. Using Luminex assay they showed that α-syn concentrations significantly decrease in the saliva of PD patients as compared to healthy controls. These findings were replicated by Al-Nimer et al. [135] and validated by ELISA assay. Vivacqua et al. [136] were able to measure, using ELISA, the concentrations of α-syn oligomer (α-syn$_{olig}$) and total α-syn (syn$_{total}$) which is constituted of α-syn monomers and to a lesser degree α-syn oligomers in saliva. They detected a significant increase in α-syn$_{olig}$ and α-syn $_{olig}$/α-syn$_{total}$ ratio in the saliva of PD patients as compared to healthy controls, while α-syn$_{total}$ exhibited a significant decrease in concentration in saliva of PD patients when compared to healthy controls. These results observed in saliva are mirrored in CSF [63]. Vivacqua et al. suggested that the difference in concentrations between α-syn$_{total}$ and α-syn$_{olig}$ was due to the oligomerization of free monomeric α-syn in saliva, which lead to the reduction of the recorded α-syn$_{total}$ concentration.

Upon analysis of the results presented by Vivacqua et al. for α-syn$_{total}$ salivary levels, it was evident that α-syn$_{total}$ is pertinent in more than simply identifying PD patients when compared to controls, as its levels on a smaller scale within the PD population exhibited correlations with disease severity, progression, stages and cognitive impairments. α-syn$_{total}$ was shown to positively correlate with H&Y scores, MDS-UPDRS total score and LEDD scores. Indicating that it can provide a tool for prediction of disease progression as lower concentrations reflect early disease stages, while higher concentrations reflect late disease stages. Negative correlations were established between α-syn$_{total}$ and both FAB and MOCA scores, indicating reduced cognitive abilities in PD patients. The increase in α-syn$_{total}$ with the advancement of disease, was attributed by Vivacqua et al. to disease progression causing advanced synaptic and cellular damage that result in α-syn monomer release into the extracellular medium. Unlike α-syn $_{total}$, α-syn $_{olig}$ was not demonstrated to have any correlation with disease stages and progression. A urinalysis study conducted on a Korean sample demonstrated that α-syn cannot be detected in urine of PD patients and healthy individuals.

DJ-1

DJ-1 is a 189 Amino Acid protein; its mutation has been associated with rare early onset familial autosomal recessive PD. DJ-1 is speculated to be a pleiotropic neuro-protective protein that functions as an antioxidant and against mitochondrial dysfunction [137–141]. Normally DJ-1 is located mainly in the cytoplasm and to a lesser extent in mitochondria and nuclei of dopaminergic neurons. Under oxidative stress DJ-1 monomers dimerize and favour mitochondrial

localization to finally translocate to the cell nucleus. Recent evidence suggests that DJ-1 recruitment to plasma membrane and mitochondria is efficient in neuro-protection only when cells are under low to moderate oxidative stress [142]. In the face of oxidative stress, DJ-1 has been shown to have the ability to reduce hydrogen peroxide species, stabilize Nrf2 transcription factor which regulates the expression of anti-oxidant proteins and reduce oxidative stress sustained by neurons upon calcium entry via L-type channels in pacemaker potentials [138,142,143]. DJ-1 also plays a role in preventing mitochondrial dysfunction through regulation of SLC25A14 and SLC25A27 which are mitochondrial uncoupling proteins in dopaminergic neurons of the substantia nigra pars compacta [142]. DJ-1 interacts with PINK1 which is a serine threonine kinase that protects cells from stress induced mitochondrial dysfunction [139,141]. There is evidence that implicates DJ-1 in regulation of astrocyte inflammatory responses as well as astrocytic and neuronal lipid rafts formation [144,145]. Finally, DJ-1 plays the role of a molecular chaperone to inhibit the formation of α-synuclein fibrils, an essential step in the formation of α-syn oligomers which constitute a key part in PD pathology [146]. Devic et al. [134] examined DJ-1 concentrations in saliva of PD patients and their respective healthy controls by Luminex assay and showed that DJ-1 levels did not correlate with clinical test scores for the UPDRS motor test, yet have a tendency to increase in PD patient saliva as compared to controls. Using the same technique, Kang et al. [147] were able to identify a significant increase in DJ-1 concentration in patients who are classified as PD stage 4 according to the H&Y score as compared to those who were classified as stages 1—3. A significant decrease in DJ-1 concentration was realized in patients with mixed type PD as compared to tremor dominant and akinetic-rigid dominant type suggesting different mechanisms of disease progression in different disease subtypes. Kang et al. reported no correlation between DJ-1 salivary concentration and UPDRS scores in accordance with the findings of Devic et al. In a recent study conducted by Masters et al. [148] and by performing quantitative immunoblotting, DJ-1 showed a significant increase in concentration and a positive correlation with UPDRS motor score and thus reflects motor disability. In turn, total salivary protein concentrations were significantly elevated in saliva of patients with respect to controls and differentiated between them. This elevation was attributed to autonomic dysfunction in PD patients. After adjusting DJ-1 levels with respect to total protein concentration, there was no difference between PD patients and controls. Similar to these studies, results concerning DJ-1 concentrations in CSF remain inconclusive [63]. A urinalysis study conducted on a Korean sample has shown that DJ-1 concentrations significantly increase in the urine of PD affected males when compared to non-PD males, while results for females remained insignificant [8].

Acetylcholinesterase activity

Dopaminergic neuron loss in PD is accompanied by a heterogeneous loss of cholinergic neurons affecting various brain regions. Cholinergic deficit with cholinergic neuron terminal reduction has been recorded in PD patients, being more severe with patients affected by PD associated dementia [149—151]. It has been established that, the decrease in gait speed associated with PD is attributed to cholinergic degeneration [152]. Recently, AChE inhibitors were shown to be effective in alleviating some PD symptoms [153]. Fedorova et al. [154] analysed AChE in saliva samples obtained from PD affected individuals and their relative controls. They were able to uncover an increase in enzymatic activity in the saliva of PD patients when compared to controls. This increase was representative of disease progression as the increase in AChE catalytic activity mirrored stage progression in the H&Y score. Federova et al. analysed total salivary protein concentration which they found to be significantly increased in PD patient saliva as a result of hyposiallorhea. The ratio AChE activity/ Total protein concentration was calculated to emphasize that the increased catalytic activity of AChE can only be attributed to an alteration in enzymatic function and not enzymatic concentration. Despite their significant differences, AChE concentrations and AChE/Total protein ratio displayed an overlap between controls and PD patients as few patients presented with values that would classify them as healthy individuals and vice versa.

Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disorder caused by an expansion of N-terminal (CAG) n trinucleotide repeat of the huntingtin gene (Htt) with locus 4p16.3. Polymorphism in the number of repeats of this trinucleotide is common among the population where repeats ranging from (CAG)9—36 are considered normal, while repeats above (CAG)37 are considered pathological [155—157]. The length of the CAG repeat has been positively correlated with early disease onset and rate of disease progression [158]. The altered CAG trinucleotide repeat codes for repeats of glutamine in the translated 3142 AA huntingtin protein. Normal huntingtin protein contributes to multiple physiological functions including embryonic development, cell survival, tissue maintenance, and cell morphology [159]. In HD, The mutant huntingtin protein is processed by proteases to release the N-terminal poly-glutamine sequence, these released fragments can interfere with transcription and trigger neurodegeneration. Also resulting from this cleavage is the C-terminal sequence of huntingtin, which mediates cytotoxicity by interfering with dynamin1 function and endoplasmic reticulum homeostasis [160]. Mutant huntingtin proteins have been shown to form cytoplasmic aggregates, whose function is still debated, as some studies find them as neuroprotective while others advocate their role in toxicity [161—163]. Neurodegeneration in HD affects multiple neuron classes in the neocortex, striatum, cerebellum, hippocampus, substantia nigra, and brainstem nuclei [164]. Usually, genetic testing for diagnostic confirmation of HD aims at identifying Htt gene mutations. Here we present Htt protein as a promising candidate for the diagnosis of HD.

Huntingtin protein

Knowing that testing CSF and blood for Htt protein for diagnostic confirmation of HD can be performed, it is still an

impractical measure due to the invasiveness of the testing procedure and because of low Htt concentrations in the acquired samples. Blood and CSF Htt protein levels remain fairly low, with a minimum requirement of a 50 ml blood sample to successfully detect Htt protein. In addition to variations in Htt protein concentrations with different blood cell types [165—167]. In a study conducted by Bloom et al. [168] Htt protein was successfully detected, using ELISA, in saliva of HD patients and healthy controls. There was a significant increase in total Htt protein concentration in saliva samples obtained from HD patients when compared to controls. Additionally, salivary concentration of mutant Htt was significantly increased in pre-manifest HD patients when compared to healthy controls. Thus, salivary Htt can serve as an early detection biomarker for HD. Given that currently a non-invasive measure of Htt CNS concentration doesn't exist. This method could facilitate diagnostic procedures or even replace existing tests once properly established.

Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a motor neuron degenerative disorder known to affect men and women in unequal ratios, approximately 2:1 [169]. The initial presentation of ALS is muscle weakness followed by progressive paralysis during the course of the disease, ultimately leading to death. ALS can also be associated with cognitive manifestations such as frontotemporal dementia [170,171]. Histologically, cellular inclusions are not an uncommon feature of neurodegenerative diseases as in Alzheimer and Parkinson. As part of its neuropathology ALS has two different types of immunohistochemical inclusions, Bunina bodies, and Ubiquitin-positive TDP-43 inclusions. The exact mode of toxicity employed by these inclusion bodies remains unknown, along with their contribution to disease pathology and progression.

ALS usually presents with upper motor neuron degeneration symptoms such as spasticity, hyperreflexia and the Hoffman sign in combination with lower motor neuron degeneration symptoms such as fasciculations, muscle cramps, and muscle atrophy [172]. These manifestations are mimicked by several other motor neuron degenerative diseases which affect diagnostic efficiency and duration of the diagnostic period. Familial ALS has been reported with genetic mutations affecting the following genes C9orf72, SOD1, TDP-43 and FUS/TLS, whereas sporadic ALS has shown mutations in SOD1, ANG, TDP-43 and TUBA4A genes. These mutations are rarely tested for, except if there exist a family history of the disease [173—176]. Because of the strenuous and unspecific nature of diagnosis, the period between disease onset, proper diagnosis and treatment initiation is prolonged, and has been established at 12—14 months [177—179]. Treatment should begin as early as possible in order to be effective, which makes early diagnosis a crucial and pressing need for ALS patients [180,181].

Chromogranin A

Chromogranin A (CgA) is a neuroendocrine secretory protein that is among the constituents of large dense core vesicles of neurons and endocrine cells containing neuropeptides and hormones respectively. Normally, CgA performs multiple physiological functions including regulation of calcium, vasoconstriction, regulation of glucose metabolism and storage, antimicrobial, antifungal and a major modulator of the neuroendocrine system [182]. Multiple studies have linked CgA to pathological features of ALS. For instance, CgA was shown to interact in a chaperone-like manner with ALS mutant SOD1 and mediate its secretion, there was a significant loss of CgA expressing neurons accompanied by decreased CgA density in the neuropil and an accumulation of CgA in the remaining neurons [183—185].

In the study performed by Obayashi et al. [186] using a YK070 chromogranin A EIA kit, salivary CgA levels were found to be only significantly elevated in patients with terminal ALS in comparison with healthy controls, moderate ALS patients and patients suffering from vascular dementia. Finally, CgA concentration positively correlated with the El Escorial score of emotional functioning rather than that of physical mobility, communication and alimentation. Thus according to these findings CgA is reflective of disease severity and the affective state of ALS patients.

Multiple sclerosis

Multiple sclerosis (MS) is a neurodegenerative, inflammatory, demyelinating disorder of the CNS with women being at higher risk than men (2:1) [187—190]. MS is believed to be an autoimmune disorder in which lymphocyte T cells target the myelin sheaths of CNS neurons [191,192]. The destruction of myelin and the breach in the BBB result in the appearance of white matter plaques, which are the hallmark of MS. The main symptoms of the disease can be sensory, motor and cognitive [193] [194—197] [198]. Psychiatric symptoms do exist in patients with MS and are manifested commonly by depression, which is a major cause of mortality in patients by increasing suicide risk [199—201]. Diagnosing MS usually relies on clinical examination, MRI scans, evoked potential testing and analysis of CSF. Because many demyelinating syndromes mimic MS in its presentation and symptoms, current MS diagnosis is performed by ruling-out these illnesses based on multiple tests and differential diagnosis techniques to confirm a diagnosis with MS [202]. This necessitates the establishment of easily accessible early diagnostic markers indicative of MS.

Soluble human leukocyte antigen, class II

The Human Leukocyte Antigen (HLA) can be grouped into two major classes, HLA class I and HLA class II both of which are coded by the Major Histocompatibility Complex (MHC) gene cluster and play an important role in antigen presentation and immune regulation which are key in eliciting an immune response [203]. Antigen presentation in MS is carried out by antigen presenting cells (APCs) which include macrophages, dendritic cells, microglia and astrocytes. These APCs endocytose myelin proteins, process them as autoantigens via the endolysosomal pathway and the resulting peptides are bound to MHC class II molecules (i.e. HLA class II). Following antigen presentation, immune cells are activated and recruited to

launch an immune response targeting the myelin sheath [204–206]. Interestingly, recent evidence has revealed a correlation between HLA mutations and levels of HLA class II molecules with MS [207–211].

The HLA classes exist in two forms, membrane bound and soluble. The membrane bound form functions in antigen presentation while the soluble form is considered as immunomodulatory and has been detected in serum, CSF, sweat, synovial fluid and saliva. The exact process by which these soluble HLA are produced or released from the membranes is still unknown. Some studies suggest that soluble HLA class I are produced by the liver and play a role in transplant tolerance; while, soluble HLA class II remain uncharacterized [212–215].

In the search for novel diagnostic salivary biomarkers for MS, Adamashvili et al. [216] determined, using ELISA, that the concentration of HLA class II is significantly elevated in the saliva of patients with Relapsing Remitting MS (RRMS) compared to healthy controls. HLA class I were not detected by the used technique, even though they are found in low concentrations in saliva. Adamashvili et al. attributed this to the low sensitivity of ELISA to low concentrations of soluble HLA class I. These results conformed to CSF measurements of both HLA classes. In accordance with Adamashvili, Minagar et al. [217] reported, using ELISA, an increase in soluble HLA class II in the saliva of patients suffering from RRMS as compared to healthy controls and undetectable salivary soluble HLA class I concentrations. They also monitored soluble HLA class II salivary levels in response to interferon β1-a, which is a medication commonly used to manage MS. Soluble HLA class II concentrations exhibited an increase in response to interferon β1-a treatment with decline in MRI contrast enhancing lesions and a stable disease course.

Oxidative stress

The release of free radicals such as reactive oxygen or nitrogen species (ROS, RNS) is normally performed during an immune response and inflammation. Cells are said to be under oxidative stress, when the released reactive species overwhelm their antioxidant defences and cause damage to cell structures that could ultimately lead to degeneration [218]. With inflammatory and autoimmune reactions being a major part in MS pathology, oxidative stress is sure to manifest in an MS affected CNS. In MS, free radicals are generated by activated macrophages, microglia and mitochondrial dysfunction [219]. Oxidative damage is known to target lipid membranes, proteins, myelin, macrophages, astrocytes, oligodendrocytes and inhibit gene expression essential for myelination. ROS also contribute to the pathological plaque formation in MS [219–221]. In the quest for establishing saliva as a reliable pool of oxidative stress markers for disease monitoring, Karlik et al. [222] performed four analytical tests on saliva and blood samples obtained from MS patients. They dosed Advanced Oxidation Protein Products (AOPP) a marker of protein oxidation, Thiobarbituric Acid Reacting Substances (TBARS) a marker of lipoperoxidation and finally advanced glycation end products (AGEs) and fructosamine which are markers of carbonyl stress. Of the four dosed oxidative stress markers, TBARS and AGEs were significantly elevated in saliva of MS

patients as compared to healthy controls, while AOPP remained unchanged. All four of the dosed markers exhibited a significant increase in plasma. Karlik et al. also assessed Total Antioxidant Capacity (TAC) and Ferric Ion Reducing Ability of saliva/plasma (FRAS/P), both of which represent saliva's and blood's antioxidant power. They found FRAS to be significantly lower in MS patient saliva samples as compared to healthy controls, while TAC levels were lower without reaching significance. The authors attributed this difference to the fact that FRAS only measures the antioxidative effects of non-protein molecules, but as the name suggests, TAC provides a total measure of antioxidative capacity in a certain fluid and is therefore affected by multiple variables. Meanwhile, plasma TAC levels decreased significantly between MS patients and controls, and FRAP remained unchanged. With the exception of one study reporting no change in CSF oxidative stress markers [223], studies conducted on CSF revealed that TBARS are increased in CSF of MS patients when compared to controls, with a decrease in TAC [221,224,225].

Autism spectrum disorders

Autism spectrum disorder (ASD) is a neurodevelopmental disorder presenting with impairments in social communication, interaction and restricted repetitive patterns of behaviour. Autistic disorder, Asperger's disorder, Rett's disorder, Childhood disintegrative disorder, Pervasive developmental disorder-Not otherwise specified-are the group of disorders that make up the ASD [226]. Despite genetic mutations being linked to the disorder and strong evidence supporting the genetic roots of ASD, there is no consensus over the specific etiology of ASD [227–229]. There exists evidence of both neurodegeneration and neuroinflammation in ASD, manifested by microglial activation, proinflammatory cytokine release and neuron loss [230,231]. Alterations in the histology and anatomy of the frontal lobe, amygdala and cerebellum have been discussed as part of the neuropathology of ASD with over-all developmental time-course of the brain being affected [232,233]. Diagnosis of ASD is based on clinical examination to identify the main symptoms of the disorder. Due to the genetic polymorphism of ASD, genetic testing is impractical to establish a diagnosis. Of the existing immunological, biochemical and hormonal biomarkers in CSF and blood for ASD, analysing blood serotonin level is the most established [234,235]. Functional MRI neuroimaging is also employed to detect differences in neurofunctional and neurophysiological activity of the ASD brain [235]. Given that early detection of ASD is important and that usually a diagnosis is not established before the age of 2 years [236–239], the search for new and easily accessible biomarkers is required that might precede the morphological and physiological changes of the disorder or help monitor the patients.

Micro-RNAs

With the ongoing search for biomarkers and causes for ASD, after identification of the genetic diversity of the disorder [227–229], research has turned to epigenetics. Epigenetics plays an important role regulating gene products without

Table 2 List of the upregulated salivary proteins obtained from ASD patients and their respective functions.

Protein	Length	Function	References
Prolactin-inducible protein (PIP)	146 AA	- IgG binding - Actin binding - Aspartic-type endopeptidase activity - Regulation of T-cell apoptosis - Regulation of immune system	[336]
Lactoferrin/Lactotransferrin (LTF)	711 AA	- Innate Immunity - Antibacterial - Antiviral - Antifungal - Immune modulator	[78,79,82,85,86]
Ig kappa chain C region (IGKC) IgG gamma-1 chain C region (IGHG1)	107 AA 330 AA	- Constant region of immunoglobulin heavy chains - Humoural immune response	
Annexin A1 (ANXA1)	346 AA	- Anti-inflammatory - Regulation of immune system - Wound repair - Chemotaxis - Neutrophil extravasation	[337−342]
Neutrophil-defensin 1 (DEFA1/DEFA1B)	94 AA	- Chemotaxis - Antimicrobial immune response - Gram positive antibacterial - Innate immune response	[343,344]
Neutrophil elastase (ELANE)	267 AA	- Calcium homeostasis - Negative regulation of chemotaxis and inflammation - Gram negative antibacterial - Down-regulation of chemokine production - Up-regulation of IL-8 production and MAP kinase activity - Transcriptional repressor	[345−350]
Lactoperoxidase (LPO)	712 AA	- Antibacterial	[351]
Lipocalin 1 (LCN1)	176 AA	- Cysteine proteinase inhibitor - Retina homeostasis	[352,353]
Polymeric immunoglobulin receptor (PIGR)	764 AA	- Transepithelial transport of immunoglobulins	[354−356]
Deleted in malignant brain tumours 1 protein (DMBT1)	2413 AA	- Carcinogenesis - Possible tumour suppressor	[357−359]
Myeloperoxidase (MPO)	745 AA	- Apoptosis - Innate immune response - Inhibits adaptive immune response - Microbicidal - Neutrophil recruitment - Oxidative stress	[360−366]

altering the nucleotide structure of the gene itself. MicroRNAs (miRNA) are short single stranded RNA sequences that interfere with gene expression by interacting with target mRNA and regulating its translation into protein. Multiple miRNA expression profiles have been elucidated in ASD most of which are involved in nervous system development and function [240−242]. In efforts to establish new diagnostic biomarkers for ASD, salivary miRNA profiling was performed by Hick's et al. [243] who was able to discover 14 differentially expressed miRNAs in ASD patient saliva when compared to healthy individuals. From these 14 miRNAs 4 were downregulated in saliva samples of ASD patients (miR-23a-3p, miR-27a-3p, miR-30e-5p and miR-32-5p) and the remaining ten were upregulated (miR-140-3p, miR2467-5p, miR-218-5p, miR-28-5p, miR-335-3p, miR-628-5p, miR-7-5p, miR-191-5p, miR-127-3p and miR-3529-3p). All miRNA levels were predictive of the Vineland Adaptive Behaviour score especially for neurodevelopmental scores except for miR-140-3p. Knowing that the Vineland Adaptive Behaviour scale is a psychometric assessment test for psychological and psychiatric disorders and that a low Vineland score indicates an impairment [244], it was interesting to find that the upregulated miRNAs negatively correlated with the Vinland score while downregulated miRNAs positively correlated with the score. Further, investigation of possible target genes for the identified miRNA revealed a vast number of enriched neurodevelopment linked and ASD linked genes. This indicates that creating a miRNA salivary profile for ASD can help properly diagnose infants

with the disorder before prominent behavioural manifestations appear.

Salivary proteome

With the immune system suspected to play an important role in ASD pathology, proteins implicated in immune reactions are expected to be deregulated. Studies regarding the ASD related neuroimmune disturbances as well as correlation of ASD with parental autoimmune disorders further verifies that the deregulation of the immune system is either etiological or consequential to ASD [231,245–249]. In the quest of establishing new ASD salivary biomarkers, Ngounou Wetie et al. [250] conducted a pilot study in which they examined discrepancies in salivary protein levels and signatures between ASD patients and healthy controls. By employing Mass Spectrometry-based proteomics, they were able to identify a variability in salivary proteomic levels for ASD with 12 proteins having an elevated concentration and 4 proteins with reduced concentration in ASD patient saliva when compared to healthy controls. The list of proteins with elevated and reduced concentrations in saliva of ASD patients along with their physiological functions are summarized in Tables 2 and 3. It is evident that all the mentioned proteins are involved in immune reactions. Further analysis of protein–protein interactions revealed that LTF and PIP both interact with prolactin. A similar analysis revealed that submaxillary gland androgen-regulated protein 3B, statherin and histatin all interact with one another. This observation led Ngounou Wetie et al. to the conclusion that protein complex formation might be compromised in ASD. In the same context, Castagnola et al. conducted a proteomic study aimed at identifying changes in the post-translational modifications of salivary proteins. They were able to detect salivary protein hypophosphorylation for ASD patients when compared to healthy controls. The four studied hypophosphorylated proteins were statherin, histatin and proline rich proteins 1 and 3. These findings support the above data indicating that

molecular changes in proteins might affect their physiological functions. Castagnola et al. [251] stated that hypophosphorylation serves more as an explanation for failed protein mechanisms rather than a biomarker and therefore could explain the essence of protein–protein interaction deficits. We can thus conclude that biochemical and physiological changes affect salivary proteins in ASD.

Oxytocin

Oxytocin is a neuropeptide produced by the hypothalamus and secreted by the posterior pituitary. It is known to function in the brain in neuromodulation, regulating the mother and infant bond, sexual behaviour and social recognition [252–254]. Studies have associated oxytocin dysfunction with multiple aspects of ASD and its administration has been found to alleviate social impairment symptoms [255–258]. In the light of the affiliation present between oxytocin and ASD two studies were performed to dose oxytocin saliva concentrations in ASD patients and compare them with healthy controls. By implementing an ELISA, Feldman et al. [259] proved that detection of salivary oxytocin was possible during early disease stages and salivary oxytocin levels in ASD patients to be significantly lower than those of normal controls. Fujisawa et al., [260] were able to dose oxytocin in saliva of both healthy controls and ASD patients by an ELISA as well. Their assay was directed at associating oxytocin salivary levels with visual attention for social signals which resulted in inconclusive evidence. Finally, a meta-analysis performed by Grazia et al. [261] combined the data of the two studies obtaining a 152 individual sample and concluded that there is no significant difference in salivary oxytocin levels between ASD patients and healthy controls. In addition to saliva, Grazia et al. conducted a meta-analysis on data obtained on oxytocin levels in serum and CSF which yielded the same results obtained from the data on saliva. Not enough data was retrieved on urine to properly conduct a meta-analysis and obtain significant results [261].

Table 3 List of the downregulated salivary proteins obtained from ASD patients and their respective functions.

Protein	Length	Function	References
Salivary acidic proline rich phosphoprotein (PRH1/2)	166 AA	- Calcium binding - Inhibition of calcium phosphate crystal formation - Inhibition of calcium carbonate precipitation - Maintenance of oral health	[367–370]
Submaxillary gland androgen-regulated protein 3B (SMR3B)	79 AA	- No known function	
Statherin (STATH)	62 AA	- Inhibition of calcium phosphate precipitation and crystal growth - Enamel boundary lubricant - Oral bacteria colonization	[371–374]
Histatin-1 (HTN1)	57 AA	- Inhibition of calcium phosphate precipitation and crystal growth - Antifungal - Wound healing - Enamel pellicle formation	[375–377]

Gene Name	Target Region	Locus	Function	Disorder	Status
5-hydroxytryptamine receptor 2A (HTR2A)	T102C polymorphic site	13q14.2	Serotonin receptor	Schizophrenia/Bipolar disorder	Hypomethylated
Dystrobrevin Binding Protein 1 (DTNBP1)	Promoter	6p22.3	Organelle Biogenesis	Schizophrenia	Hypermethylated/ Down-regulated
Membrane-Bound Catechol-O-Methyltransferase (MB-COMT)	Promoter	22q11.21	Catecholamine Neurotransmitter degradation	Schizophrenia/Bipolar disorder	Hypomethylated/ Up-regulated
Vasoactive Intestinal Peptide Receptor 2 (VIPR2)	CpG	7q36.3	G-protein coupled receptor for vasoactive intestinal peptide	ADHD	Hypomethylated

Table 4 Results of salivary epigenetic methylation profile of patients suffering from schizophrenia, bipolar disorder and ADHD. The table below provides information concerning the target region of the epigenetic modification. The chromosomal localization of the gene. The function of the coded protein. As well as the methylation status of the gene for the respective neuropsychiatric disorder.

Neuropsychiatric disorders

Schizophrenia, bipolar disorder and attention deficit hyperactivity disorder (ADHD) all fall under the title of neuropsychiatric disorders, which present with multiple behavioural symptoms. Symptoms of schizophrenia are grouped into: (i) Positive — delusions and hallucinations; (ii) Negative — reduced motivation, social impairments, reduced pleasure and blunted affect; and finally (iii) Disorganized speech and behaviour [262−264]. Bipolar disorder is classified as a major mood disorder with symptoms of varying degrees of mania and depression [265]. Aside from the multitude of social impairments that affect patients, a high suicide risk has been correlated with both schizophrenia and bipolar disorder [266,267]. Of the three mentioned disorders only ADHD is classified as a childhood disorder. ADHD is characterized with severe persistent debilitating hyperactivity that leads to functional and social impairments [265]. A diagnosis is usually established for these disorders after proper clinical examination and observation based on the aforementioned symptoms and other DSM-V criteria [226]. Genetic causes have been associated with all three diseases, but the specific etiology remains to be elucidated [268−273]. Given that diagnosis of the Neuropsychiatric disorders relies on clinical examination of the patient without the establishment of specific disease biomarkers, exploration of physiological fluids for easily accessible and accurate biomarkers seems essential in efforts to provide innovative and faster diagnostic processes.

DNA Methylation

Multiple genes can be named as players in causing the aforementioned neuropsychiatric diseases, as there is no single culprit capable of explaining the various symptoms and types of the disorders [268−273]. Because of this, research has turned its attention towards the possible implication of aberrant epigenetics in causing the pathological phenotypes associated with neuropsychiatric disorders [274−279]. On this basis, investigation of methylation profiles of salivary DNA of schizophrenic, bipolar and ADHD patients allowed the identification of four genes HTR2A, DTNBP1, MB-COMT and VIPR2 [Table 4] with epigenetic alterations when compared to normal controls [280−283]. Cumulative research efforts are providing mounting evidence for the involvement of HTR2A, DTNBP1 and MB-COMT in both schizophrenia and bipolar disorder [284,285]. Deregulation of both serotoninergic and dopaminergic systems have been shown, with these genes known as major players in their pathways [286−289]. Pathological genetic variants of HTR2A, DTNBP1 and MB-COMT were proven to affect memory [290−292], hallucinations [293], glutamate signalling [291], and symptom severity [294,295]. Reports of correlations between VIPR2 methylation and ADHD may be premature and thus the function of this gene in ADHD requires further studies [296,297]. In fact, the study conducted by Wilmot et al. [283], who detected VIPR2 CpG methylation in saliva, was the first of its kind performing a genome wide DNA methylation analysis for ADHD. Aside from the downregulation of DTNBP1, the study conducted by Abdolmaleky et al. [280] characterized the level of gene

methylation as an indicator to treatment responsiveness for both schizophrenic and bipolar subjects. Results indicated that patients undergoing and responding to treatment had lower levels of DTNBP1 promoter methylation as compared to untreated patients. The disease-associated hypermethylation was detected in early schizophrenic patients and in healthy first degree relatives, with higher levels of methylation associated with early disease onset. This data supports the merit of DTNBP1 in disease prediction and treatment management. Similar to DTNBP1, HTR2A hypomethylation was also detected in first degree relatives, suggesting the heritability of the epigenetic anomaly [281]. In addition, by comparing salivary samples to post-mortem brain samples, the salivary methylation status of MB-COMT, DTNBP1 and HTR2A was reported to mirror the brain's methylation status for these genes.

Salivary proteome

Similar to the discussed Neurodegenerative diseases, immune system deregulations have been put into evidence for bipolar disorder and schizophrenia [298–301]. Thus it is only logical that levels of proteins involved in eliciting an immune response ought to be altered in schizophrenic and bipolar patients when compared to healthy controls. From this standpoint, Iavarone et al. [302] conducted a salivary proteome analysis and found eight immune system-related proteins to be elevated in the saliva of schizophrenic and bipolar patients when compared to healthy controls. The identified proteins were α-defensins 1–4, S100A12, cystatin A and the S-derivatives of cystatin B (glutathionylated and cysteinylated). All were significantly increased in both disorders except for α-defensin 3 in bipolar disorder, where it exhibited a slight non-significant decrease with respect to the controls. All of the mentioned proteins are involved in innate immunity and therefore indicate an immunologic imbalance in schizophrenic and bipolar patients [303–306].

Discussion

Early detection of neuro-degenerative and neuropsychiatric disorders is imperative for better disease prognosis and the initiation of early treatment [4]. The two main physiological fluids that are used for the detection of such markers are CSF and blood. Acquiring samples of these two fluids has proven to cause a certain level of discomfort and pain to the patient [5]; therefore, it is essential to establish a substitute that is less invasive but remains representative of the body's physiological changes. A good candidate offering promise as a biomarker pool for neurological disease diagnosis and monitoring is saliva. It is an easily accessible biological fluid and its collection is non-invasive, painless and cost effective [14]. In addition to its ease of collection, saliva is generally safer than blood and CSF, and its collection does not expose the healthcare provider to needles, thus reducing the risk of pathogen transmission from patients suffering from chronic infection. Similar to saliva, urine seems to provide a novel and non-invasive source of biomarkers for neurological disorders.

Following the genetic findings provided by Lee et al. [52] and Bermejo Pareja et al. [47], who reported associations between Aβ42 concentration in saliva with AD patient medical history and genetic data, it could be concluded that salivary Aβ42 is probably more reflective of familial AD genotype rather than sporadic AD, and can be used as a tool for disease prediction but requires further investigation. Based on the differential detection of Aβ42 between groups, where it has been found to be secreted in high concentrations in the saliva of individuals suffering from or at risk of developing AD, as compared to healthy controls and controls suffering from PD, Aβ42 can be considered as a prominent candidate as salivary biomarker for AD; however, a gold standard test must be developed for its reproducible and accurate detection in saliva. Further investigation must be carried out in larger population samples to identify the exact diagnostic concentration ranges for salivary Aβ42. Moreover, there is a crucial need to validate the differential detection of Aβ42 in different disease stages. Unlike Aβ42, analysis of Aβ40 has produced conflicting and insignificant findings [47,52]. This lack of evidence renders it less reliable as a salivary biomarker for AD diagnosis or disease staging without further evaluation. Similar to Aβ42, tau pathology has been reported in Parkinson's disease [63]. Therefore, further investigation with different detection techniques and the inclusion of Parkinson controls must be performed to confirm the validity and potential use of p-tau/t-tau as a biomarker for AD. As described by Carro et al. [77], lactoferrin is highly representative of AD pathophysiology, cognitive impairment and phenotypes and therefore qualifies as a promising salivary biomarker for disease diagnosis. Further longitudinal studies and comparative studies enrolling individuals suffering from multiple cognitive impairments are required to establish the true validity and diagnostic merit of lactoferrin for AD. The weakness of the study conducted by Haixiang Su et al. [96] on protein carbonyls as biomarkers for AD lies in the age difference between controls (mean = 69.20) and AD patients (mean = 82.4) which necessitates a new study with age adjustment among the compared groups for more accurate findings.

Based on the reports on α-syn and DJ-1 as PD biomarkers [134–136,147,148], it could be inferred that the two molecular forms of α-syn, oligomeric and monomeric, can be considered in the search for salivary biomarkers for PD. With α-syn olig serving as a biomarker for disease diagnosis only, due to the lack of data that links it to disease stage and prognosis, and α-syn total serving as a staging biomarker to track disease progression. DJ-1 could be considered as a salivary biomarker for diagnosing the disease and identifying the type of Parkinsonism.

The observations reported on salivary AChE activity in both AD and PD patients [72–74,154], indicate that at present salivary AChE cannot be used as a diagnostic marker. In light of present research, AChE could be considered only for monitoring disease pathophysiology and parasympathetic denervation in PD. Properly designed and multifaceted studies must be conducted in order to confirm the potential use of salivary AChE in AD and PD diagnosis. These studies should take into account the various disease stages (mild, moderate, and severe) and treatment programs of every patient, as well as the development of a proper technique for sample collection and storage for maintaining optimum enzyme function.

Due to their involvement in immune system reactions, peptides, oxidative stress markers and soluble HLA II
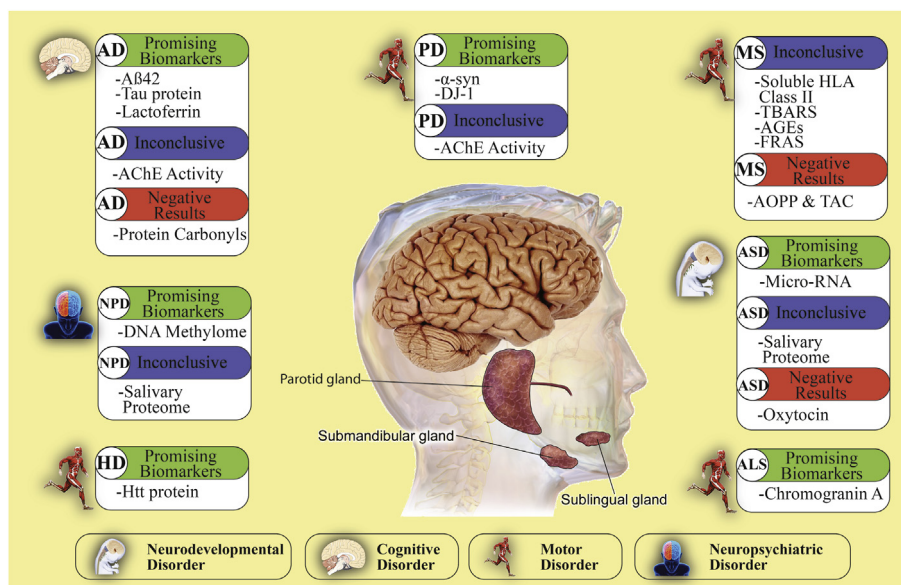
Fig. 2 **Summary of salivary biomarkers and their current state of validity**. This figure classifies the neurological disorders into four different types: neurodevelopmental, cognitive, motor and neuropsychiatric disorders. For each one of these disorders, a categorical system classifies the biomarkers as: promising, inconclusive and negative results based upon the findings of our work. Abbreviations used: AD: Alzheimer's disease; PD: Parkinson's disease; MS: Multiple Sclerosis; ASD: Autism Spectrum Disorder; ALS: Amyotrophic Lateral Sclerosis; HD: Huntington's disease; and NPD: Neuropsychiatric Disorders.

remain inconclusive as predictive biomarkers, pending further investigation. It is crucial to conduct comparative studies between neurological conditions that trigger the immune system and other immunological conditions, in order to establish whether these markers are exclusively indicative of neurological conditions or can be detected in and affected by the presence of other immunological deregulations.

Investigations on salivary biomarkers are still inconclusive because there is not enough evidence to support or negate the actual pertinence of these molecules in saliva. Most of the existing studies were based on relatively small samples that lacked sufficient power, in addition to concluding with few conflicting results. Based on the results presented by our review, we realize that there are some biomarkers which are more prominent than others, especially those proven to be causal for disease phenotypes, while those associated with other physiological processes like immunity and oxidative stress remain somewhat unspecific unless their presence in saliva is proven to be strictly representative of the diseases in question [Fig. 2]. The presence of the reviewed salivary biomarkers detected in neurological conditions was also assessed in different commonly tested physiological fluids: CSF, blood and urine [Table 5], along with a comparison of each fluid's advantages and disadvantages [Table 6].

That being said, we cannot depend on one biomarker to ultimately achieve an accurate diagnosis. Instead, a definitive biomarker profile must be created to be used for future diagnostic purposes. The source of these biomarkers remains to be properly identified. They could be released into saliva from plasma microfiltration through the gums or expressed in salivary glands. It is also probable that they are transported directly from the CNS via the axons of the facial and glossopharyngeal nerves to the salivary glands to ultimately be vacated along with saliva secretion.

Because different fundamental and prominent factors may affect sample quality, saliva collection methods and processing must be refined. In the study conducted by Lee et al., stabilizing salivary levels of Aβ42 peptides by adding thioflavin S to prevent aggregation and using sodium azide to inhibit bacterial growth, enhanced Aβ42 detection in the sample [52]. Furthermore, the available literature indicates that patients suffering from neurological disorders have lower oral health and are more prone to oral disease than healthy individuals [307–313]. We thus speculate the possible interference of reduced oral health and successful biomarker detection. This raises the question if proper extraction, handling and treatment of the saliva sample with agents and compounds known to stabilize the measured molecule will result in better sample quality and therefore better biomarker detection.

Another aspect of saliva that might affect proper biomarker detection is the fact that patients suffering from some of the neurological diseases listed in this review suffer from sialorrhea or hyposialorrhea which are excess and diminished salivation respectively. The changes in saliva secretion is usually brought on as a side effect of the illness [314–318] or caused by medication [317,319,320]. This indicates that there is a possibility of shifts in biomarker concentrations among healthy controls and patients due to different secreted concentrations of total salivary proteins. Consequently, it is recommended to normalise future measurements of salivary biomarkers against total salivary

Table 5 Summary of available reports on the levels of disease biomarkers detected in saliva, CSF, blood and urine and their respective variations.

		Saliva	CSF	Blood	Urine
AD	AB42	↑ in Moderate AD [47] ↓ in Severe AD [47] ↑ [51,52]	↓ in Severe AD [48–50,378,379]	↑ [380,381] ↓ [382,383]	Detected [9]
	AB40	↑ [51] No change [47]	No change [378,379]	↑ [378,380,384] ↓ [385]	N/A
	Tau	↑ [13]	↑ [63,386,387]	No change [384]	N/A
	Lactoferrin	↑ [77]	N/A	No change [388,389]	N/A
	AChE activity	↓ [72–74]	↑ in AD with APOE4 mutation [390] No change [391] ↓ [391]	↓ Erythrocyte AChE activity [392] No change [391]	N/A
	Protein Carbonyls	No change [96]	No change [393]	↑ [394–397]	N/A
PD	α-syn$_{total}$	↓ [136,398] ↑ [134,135]	↓ [63] No change [63]	↓ [399] ↑ [400,401]	Not detected [8]
	α-syn$_{olig}$	↑ [136]	↑ [63]	N/A	N/A
	α-syn$_{olig}$/α-syn$_{total}$	↑ [136]	↑ [63]	N/A	N/A
	DJ-1	↑ [134,147,148]	↓ [398] ↑ [402,403]	↑ [404] No change [405,406]	↑ [8]
	AChE activity	↑ [154]	No change [391]	↑ [407]	N/A
HD	mHtt protein	↑ [168]	↑ [167]	↑ [165,166]	N/A
ALS	CgA	↑ [186]	↑ [408]	N/A	N/A
MS	s-HLA Class II	↑ [216]	↑ [216] Detected [409]	↑ [210] ↓ [409]	N/A
	TBARS	↑ [222]	↑ [221,224,225] No change [223]	↑ [221–224] No change [225]	N/A
	AOPP	No change [222]	N/A	↑ [222]	N/A
	AGEs	↑ [222]	N/A	No change [222]	N/A
	TAC	No change [222]	↓ [221,224]	↓ [222]	N/A
	FRA	↓ [222]	N/A	No change [222]	N/A
	Fructosamine	N/A	N/A	↑ [222]	N/A
ASD	Oxytocin	No change [261]	No change [261]	No change [261]	N/A

All variations reported are in comparison to healthy controls, Legend: ↑: increasing, ↓: decreasing, N/A: no studies found, Detected: no information concerning the concentration only that the biomarker is detected, If biomarker variation is specific to disease stage, phenotype or cell type, then it is clearly stated.

Table 6 Advantages and disadvantages of sampling saliva, CSF, blood and urine.

	Saliva	Urine	Blood	CSF
Advantages	- Non-invasive - Painless - Cost effective		- Highly representative of internal physiology	
Disadvantages	- Diurnal variation - Variable volume - Variable biomarker concentration - Possible blood contamination - Oral healthrowhead	- Urinary tract infection	- Moderately invasive - Drowsiness - Discomfort	- Invasive - Pain & Discomfort - Possible allergic reaction to the anaesthetic - Infection - Headache - Dizziness - Vomiting

proteins to account for shifts in concentrations of biomarkers brought about by irregular salivation. Aside from pathological changes in salivary composition and secretion, it is established that there exist circadian rhythms that normally govern saliva production, composition and flow rate [321]. Thus it is imperative that samples from patients and controls be collected during the same time frame in order to avoid normal shifts in saliva protein concentrations from confounding the evidence.

We propose that future research efforts be directed towards establishing salivary epigenetic signatures based on micro-RNA analysis. Given that micro-RNA can be successfully detected in human saliva [322] and that micro-RNA disruptions do exist in neurological disease [323,324], it is possible to create disease specific micro-RNA expression profiles. These profiles will allow detection of a pathological fingerprint unique to the disease in question and therefore allow for a better, more accurate diagnosis.

Conclusions

Neurological disorders are generally debilitating for patients and they impose a socioeconomic burden on these patients and their caregivers. This highlights the urgent requirement for the development of easily accessible, non-invasive and cost effective diagnostic tests that aim at early identification of neurological diseases. Saliva is a physiological fluid that shows promise in developing non-invasive testing for disease biomarkers, but the utility and applicability of salivary biomarkers in diagnosis of neurological disorders remains in question. The current research remains inefficient in discerning the reliability of salivary biomarkers in neurological disease detection and monitoring. Due to the lack of data and conflicting results of the existing studies, there is an incessant need for increased research efforts to conduct well-structured clinical trials if a non-invasive screening technique based on salivary biomarkers is to be realized. Proper saliva collection and processing protocols must be standardized in order to decrease biases and allow an accurate identification of salivary biomarkers. Furthermore, we believe that salivary biomarkers, in their initial stages, will not be able to solely detect neurological pathologies, but instead these biomarkers will serve as means aiding in diagnosis, or simply replacing other invasive tests. From this standpoint and based on the reviewed literature, we were able to categorise the available biomarkers [Fig. 2], in hope of guiding future research endeavours along a targeted path to ascertain the validity of the most promising biomarkers available.

Conflicts of interest

The authors declare no conflict of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at https://doi.org/10.1016/j.bj.2018.03.004.

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