A systematic review of salivary biomarkers in Parkinson's disease

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From the Contents

Abstract

The search for reliable and easily accessible biomarkers in Parkinson's disease is receiving a growing emphasis, to detect neurodegeneration from the prodromal phase and to enforce disease-modifying therapies. Despite the need for non-invasively accessible biomarkers, the majority of the studies have pointed to cerebrospinal fluid or peripheral biopsies biomarkers, which require invasive collection procedures. Saliva represents an easily accessible biofluid and an incredibly wide source of molecular biomarkers. In the present study, after presenting the morphological and biological bases for looking at saliva in the search of biomarkers for Parkinson's disease, we systematically reviewed the results achieved so far in the saliva of different cohorts of Parkinson's disease patients. A comprehensive literature search on PubMed and SCOPUS led to the discovery of 289 articles. After screening and exclusion, 34 relevant articles were derived for systematic review. Alpha-synuclein, the histopathological hallmark of Parkinson's disease, has been the most investigated Parkinson's disease biomarker in saliva, with oligomeric alpha-synuclein consistently found increased in Parkinson's disease patients in comparison to healthy controls, while conflicting results have been reported regarding the levels of total alpha-synuclein and phosphorylated alpha-synuclein, and few studies described an increased oligomeric alpha-synuclein/total alpha-synuclein ratio in Parkinson's disease. Beyond alpha-synuclein, other biomarkers targeting different molecular pathways have been explored in the saliva of Parkinson's disease patients: total tau, phosphorylated tau, amyloid-β1–42 (pathological protein aggregation biomarkers); DJ-1, heme-oxygenase-1, metabolites (altered energy homeostasis biomarkers); MAPLC-3beta (aberrant proteostasis biomarker); cortisol, tumor necrosis factor-alpha (inflammation biomarkers); DNA methylation, miRNA (DNA/RNA defects biomarkers); acetylcholinesterase activity (synaptic and neuronal network dysfunction biomarkers); Raman spectra, proteome, and caffeine. Despite a few studies investigating biomarkers targeting molecular pathways different from alpha-synuclein in Parkinson's disease, these results should be replicated and observed in studies on larger cohorts, considering the potential role of these biomarkers in determining the molecular variance among Parkinson's disease subtypes. Although the need for standardization in sample collection and processing, salivary-based biomarkers studies have reported encouraging results, calling for large-scale longitudinal studies and multicentric assessments, given the great molecular potentials and the non-invasive accessibility of saliva.

Key Words: alpha-synuclein; amyloid-beta; autophagy; DJ-1; neurodegeneration; neuroinflammation; Parkinson's disease; salivary biomarkers; tau

Introduction

Among neurodegenerative disorders, Parkinson's disease (PD) is the fastest growing in incidence, disability, and deaths (GBD 2016 Neurology Collaborators, 2019). In PD, progressive neurodegeneration affects different regions of the central and peripheral nervous system, underlying a wide spectrum of motor and non-motor symptoms (Braak et al., 2003, 2006). Currently, the diagnosis of PD predominantly relies on clinical criteria, focusing on the presence of cardinal motor symptoms (Postuma et al., 2015). However, motor symptoms are clinically evident after a long-lasting prodromal phase (Bloem et al., 2021). This suggests that what is currently identified as the early clinical stage, is instead indicative of a pathological process that began years, if not decades, before the clinical diagnosis (Bloem et al., 2021). Given this discrepancy between clinical presentation and underlying

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pathology, there is a growing emphasis on the development of early, easily accessible diagnostic and prognostic biomarkers, able to intercept the heterogeneous trajectories of PD neurodegeneration from the prodromal phase to the advanced stages of the disease.

Alpha-synuclein (α-syn), the main component of Lewy bodies and Lewy neurites, is widely accepted as a histopathological hallmark of PD (Spillantini et al., 1997, 1998). α-Syn misfolding with the formation of α-syn oligomers represents the main responsible for α-syn neurotoxicity and cell-to-cell transmission of α-syn pathology (Spillantini et al., 1998; Cheng et al., 2011). Indeed, a crucial property of α-syn oligomers is their ability to propagate from one neuronal cell to the other, exploiting the synaptic connections in a prionic-like fashion (Olanow and Brundin, 2013; Jan et al., 2021). For this reason, α-syn oligomers represent the ideal biomarker candidate for both diagnosis and progression of PD.

However, a variety of biological mechanisms contribute in a synchronous and integrated manner to the pathogenesis of PD, spanning from altered energy homeostasis, cell death, or altered proteostasis to neuroinflammation, perturbation of cellular lipids pathways, DNA or RNA defects, and synaptic and axonal dysfunction (Bengoa-Vergniory et al., 2017; Shahmoradian et al., 2019; Wilson et al., 2023). Contribution of each mechanism may vary among different affected individuals, underlying the heterogeneity in clinical presentation and disease progression observed in PD patients. Detection of biomarkers related to different molecular pathways could therefore improve the patient's molecular stratification, tailoring a personalized approach to disease-modifying treatments.

To identify biomarkers for PD, researchers are exploring both cellular and molecular mechanisms and potential peripheral anatomical sites for detection. Aggregated α-syn has been widely detected in the autonomic small fibers innervating the skin (Wang et al., 2020a; Donadio et al., 2021; Gibbons et al., 2023) or the salivary glands (Iranzo et al., 2018; Campo et al., 2019; Manne et al., 2020; Mangone et al., 2022), which represent suitable sources for detection of α-syn pathology *in vivo*. However, biopsies from the submandibular gland and minor salivary glands have shown inconsistent results in PD and are invasive (Cersósimo et al., 2011; Folgoas et al., 2013; Iranzo et al., 2018; Ma et al., 2019). Skin biopsies, while showing high diagnostic accuracy, are also invasive and require further standardization (Wang et al., 2020b; Donadio et al., 2021; Mammana et al., 2021; Giannoccaro et al., 2022; Nolano et al., 2022; Oizumi et al., 2022). Cerebrospinal fluid (CSF) has been a primary focus (Parnetti et al., 2019; Grossauer et al., 2023), but its collection is invasive and can be contaminated by blood, affecting the reliability of α-syn detection (Shi et al., 2010; Paciotti et al., 2021). Blood samples also present challenges due to potential hemolysis of red blood cells, which contain large amounts of α-syn (Shi et al., 2010; Paciotti et al., 2021). Recent studies on neural-derived extracellular vesicles in blood samples showed promising results, but their source and specificity are still under debate (Agliardi et al., 2022). Lastly, tests on the nasal brush of the olfactory mucosa have yielded inconsistent results, even though the collection method is non-invasive and standardized (De Luca et al., 2019; Stefani et al., 2021; Bongianni et al., 2022).

In comparison to the other tissues and biofluids, saliva represents an intriguing and advantageous biofluid to investigate potential diagnostic and prognostic biomarkers of PD. Above all, saliva sampling is easily collectable and almost free of blood contamination. Furthermore, saliva, as a complex biofluid, contains a variety of molecules, which can be tested as biomarker candidates also towards a multi-omic approach. Moreover, an increasing body of evidence supports the good accuracy and reliability of the results obtained in saliva.

In this review, we first depict the biological and morphological scenario on which the detection of biomarkers in saliva is founded. Thereafter, we systematically review the previous studies investigating PD biomarkers in saliva, reporting the main achievements to date and discussing future directions for research implementation.

Morphological and biological bases of salivary glands and salivary secretion

Anatomy and histology of the salivary glands

The three paired major salivary glands are the parotid gland, the sub-mandibular gland, and the sub-lingual gland. The parotid glands are purely serous; in fact, they produce a watery, protein-rich secretion with a large amount of amylase and lipase (Jensen Kjeilen et al., 1987). The submandibular gland is a mixed gland, since it presents both serous and mucous acini (Elishoov et al., 2008). Lastly, the sublingual gland is the smallest of the major salivary glands. It secretes the smallest portion of saliva per day, 7–8%, and it represents another mixed gland, but predominantly with mucous acini (Becerra et al., 2003). Together with the major salivary glands, there are numerous minor salivary glands (ranging from 600 to 1000 throughout the mouth), located mainly in the labial, buccal, palatal, lingual, and retromolar regions.

Histologically, the secretion of saliva happens thanks to a pool of secretory vesicles clustered at the apical domain of the salivary secretory cells and releasing their content in the lumen of the acini. The process of exocytosis of the salivary vesicles follows the steps of docking, priming, and fusion and it is regulated by intra-cytosolic calcium in a way very similar to the synaptic vesicles (Fujita-Yoshigaki, 1998; Yoshimura et al., 2002; Messenger et al., 2014). More interestingly, VAMP-2, a protein interacting with the SNARE complex in the synaptic vesicles, has been also reported as involved in the secretion of the salivary vesicles (Fujita-Yoshigaki, 1998; Wang et al., 2007). After being released in the lumen of the acinus, the primary saliva proceeds towards the striate intercalated ducts, before conveying into the system of excretory ducts. Intercalated ducts operate a modification of the salivary composition by an active reabsorption of electrolytes and solutes, which employ the activity of a huge amount of mitochondria, lining the membrane of the ductular cells, in proximity with both basal and luminal domain (Tandler et al., 2006).

Salivary-secreting cells are hugely innervated by autonomic fibers. Parasympathetic innervation arrives from the superior

and the inferior salivatory nuclei and respectively travels with the intermediate nerve (VII C.n.) and the glossopharyngeal nerve (IX C.n.) as preganglionic fibers, reaching the submandibular ganglion for the innervation of submandibular and sublingual gland and the otic ganglion for the innervation of the parotid gland. Parasympathetic postganglionic fibers are cholinergic fibers, co-expressing substance P as a co-transmitter (Avery et al., 2001). They typically interact with the secretory cells and the ductular cells in an epilemmal fashion (with the fibers placed in the connective stroma around the secretory cells) (Garrett and Kidd, 1993). Sympathetic innervation arises from the intermedio-lateral nuclei of the thoracic spinal cord (segments T1–T3). Postganglionic neurons are noradrenergic and are placed in the superior cervical ganglion. Postganglionic fibers reach the salivary glands through a nervous plexus around the branches of the external carotid artery. Interestingly, the relation of the sympathetic fibers with the secretory cells is hypolemmal and the nervous fibers are placed between two cells in strict contact with the plasma membrane of the secretory cells (Garrett and Kidd, 1993). Hypolemmal autonomic fibers, taking contact with salivary-secreting cells and with cells of the salivary ducts, can release neuronal-derived vesicles in the context of the saliva and therefore, saliva could be a source of biomarkers directly derived from the autonomic nerve fibers. Moreover, different alternative anatomical pathways have been described to connect saliva with the brain, including the oral microbiota, the lymphatic-immune pathways, the olfactory pathways, or the peripheral blood stream, which may create a complex salivary-brain axis (Zürcher and Humpel, 2023). Biology of salivary secretion, relevant for biomarkers implementation in PD, is highlighted in **Figure 1**.

Biochemical and physical properties of saliva

Saliva is a complex and dynamic biological fluid, which is mainly composed of a high percentage of water (roughly 95%) and a variety of electrolytes. Moreover, a plethora of other molecules are detectable in saliva and includes proteins and glycoproteins, RNAs, lipids, and different metabolites, such as urea and ammonium, or products of the catabolism of a variety of bioactive molecules, including neurotransmitters. Together with these purely molecular components, saliva also contains living cells (lymphocytes) or cell fragments (desquamated mucosal cells and fragments of salivary secretory cells), which could be considered themselves a further source of possible biomarkers, such as cytokines, phospholipids derived from the biological membranes, trans-membrane proteins or DNA (Humphrey and Williamson, 2001). The different salivary components interact between them to achieve the general functions of saliva (Levine, 1993a, b; Dowd, 1999).

The average daily flow of whole saliva varies in health between 1 and 1.5 L. Contributions of the different salivary glands during unstimulated flow account respectively as follows: 20% for parotid, 65% for submandibular, 7% to 8% for sublingual, and less than 10% from the widespread minor glands. Contribution of the different salivary glands differs also in terms of molecular products. The Parotid gland produces mostly amylase, proline-rich proteins, agglutinins, and IgA, with reduced amounts of cystatins, lysozymes, and

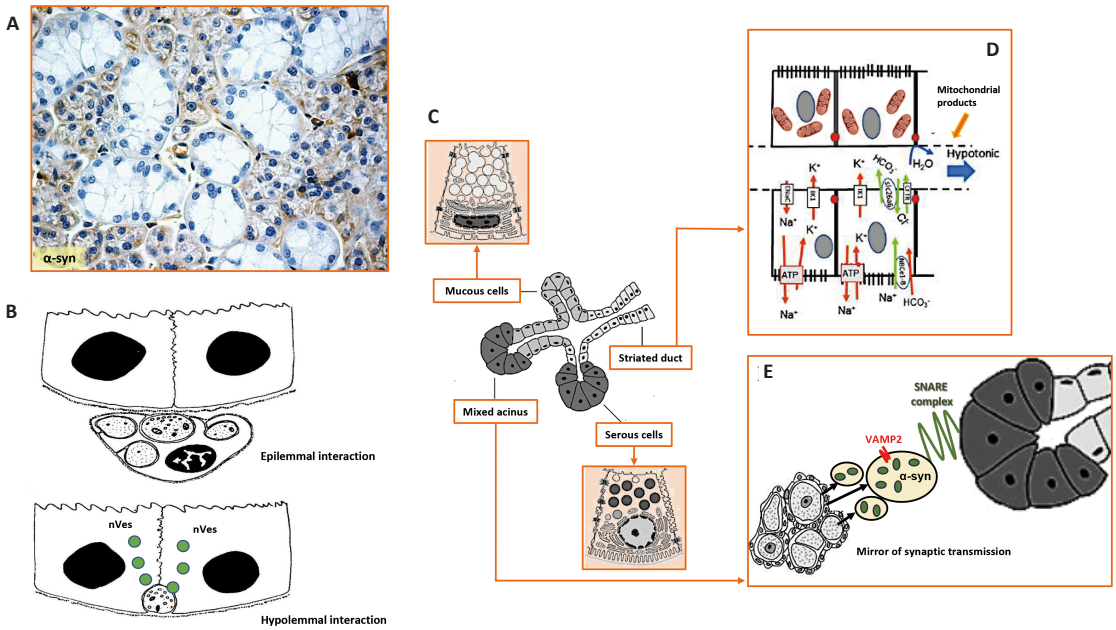

Figure 1 | **Salivary glands are richly innervated by sympathetic and parasympathetic fibers which are distributed around the secreting cells of the acini.**
As demonstrated by immunohistochemistry, on a healthy submandibular gland (A), alpha-synuclein (α-syn) is strongly present in nervous fibers around salivary-secreting cells, with a prevalence for serous acini rather than mucous tubules. The two possible anatomical interactions of the nervous fibers with the salivary-secreting cells are represented in (B), where it is also highlighted that the exchange of neuronal-derived vesicles (nVes) can occur between nervous fibers and salivary-secreting cells, especially in case of hypolemmal relation. In (C), the anatomical and histological organization of a salivary acinus is reported, with a detail of the cytology of serous and mucous cells. Protein secretion by the salivary acinar cell is associated with increases in [cAMP]i and [Ca^{2+}],. Activation of cAMP via the contribution of β-adrenergic, muscarinic, and substance P receptors is a key modulator of salivary secretion. The primary secretion of the salivary acinar cell is isotonic, but it becomes hypotonic by the passage in the striated ducts, where electrolytes are reabsorbed thanks to active transport (D) and where ductal epithelial cells are rich in mitochondria, able to reverse in saliva different products including oxidative metabolites. On the other hand, the secretion of saliva follows an exocytotic mechanism very similar to the secretion of synaptic vesicles and previous studies have reported the contribution of the SNARE complex to the secretion of saliva in a way similar to that involved in synaptic transmission (E). Created with Microsoft PowerPoint. cAMP: Cyclic adenosine mono-phosphate; SNARE: SNAP (soluble N-ethylmaleimide-sensitive factor attachment protein) receptor; VAMP2: vesicle-associated membrane protein 2.

glycoproteins. Sublingual saliva contributes highly to the secretion of both types of mucins: high molecular weight mucins (MG1) and low molecular weight mucins (MG2), together with high levels of lysozymes and ribozymes. Submandibular saliva contains the largest amount of cystatins and histatins, whereas palatine and lingual minor salivary glands predominantly secrete MG1 mucins and a relatively high amount of amylase (Levine, 1993b; Veerman et al., 1996). Major salivary glands are also responsible for the secretion of water and electrolytes.

Generation of saliva occurs as an ultrafiltrate of the plasma enriched by the specific salivary proteins (Humphrey and Williamson, 2001). Primary saliva, produced by the acinar cells, is isotonic with the plasma, but traveling along the striated salivary ducts, it becomes progressively hypotonic. Hypotonicity, especially when an increased salivary flow occurs, allows for expansion and hydration of mucin glycoproteins, which represent a protective blanket for the oral mucosa (Tabak et al., 1982).

The physiological pH of saliva is slightly acidic and ranges between 6 and 7. However, variations in pH from 5.3 (low flow) to 7.8 (peak flow) could depend on the salivary flow and the variations of the saliva composition.

Interestingly, salivary secretion is regulated and stimulated by several factors, which can also vary among different individuals. Various neurotransmitters and hormones modulate in different ways the various salivary-secreting cells, eliciting a variety of responses in each of them. In particular, sympathetic innervation induces protein secretion, whereas parasympathetic stimulation increases the content of water and electrolytes, making saliva more diluted and with lower oncotic pressure (Culp et al., 1991). Moreover, there is great individual variability in the modulation of salivary secretion, thereby changing the molecular composition of saliva in different individuals. Finally, daily and annual ebbs and peaks in salivary flow also occur. Circadian (daily) low flow occurs during sleep, whereas peaks occur during high stimulation periods. Circannual (yearly) low flow occurs during summer, whereas peak flow happens during the wintertime. These reasonably suggest potential variations in the molecular composition of saliva during the day and different periods of the year (Dawes, 1974; Rudney, 1995).

Methods

This systematic review has been performed according to the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) Statement and following the PRISMA checklist (Page et al., 2021).

Two reviewers (MIDB and MC) separately performed study eligibility criteria, search strategy, selection process, data extraction, and quality assessment.

Eligibility criteria

All peer-reviewed original articles in the English language investigating salivary biomarkers in adult PD patients in comparison to healthy controls (HC) and/or to patients with neurodegenerative disorders other than PD have been considered eligible. Exclusion criteria were: animal studies, conference abstracts, review articles, case reports/series, clinical trials, and studies not pertinent to our objectives.

Search strategy

Literature searches were conducted on both PubMed and SCOPUS databases, focusing on publications available up to September 2023. For PubMed, the search string used was: ("Parkinson's Disease" [Title/Abstract] OR "Parkinson" [Title/Abstract]) AND ("saliva"[Title/Abstract] OR "salivary"[Title/Abstract]) AND ("biomarker"[Title/Abstract] OR "marker"[Title/Abstract]). A similar approach was employed for the SCOPUS database, ensuring consistency in the search criteria across both platforms. No filters were set during the search process.

Selection process, data extraction, and quality assessment

All search results were aggregated in Excel for Windows. Initially, duplicates were identified and removed, ensuring that unique references were retained. Subsequently, the studies were screened based on their titles and abstracts. Studies that met the predefined inclusion and exclusion criteria were included. Relevant articles were thus selected for full-text review. Articles were also selected for full-text review if eligibility could not be ascertained solely from the title or abstract.

In case of potential discrepancy regarding eligibility criteria and selection process, a consensus meeting was held with other co-authors to resolve any differences and reach an agreement. The entire literature selection process is summarized in **Figure 2**. Data were extracted using a standardized data extraction form. Extracted data included: first author, year of publication, number of patients (PD and controls), types of biomarkers, measurement methods, mean findings, sensitivity, specificity, and accuracy of the results. From studies comparing different biofluid samples, only results from saliva samples have been extracted. Results from PD in comparison to other neurodegenerative disorders (i.e. AD or atypical parkinsonisms) have been reported to evaluate the specificity and accuracy of the different biomarkers. Selected studies were divided into subgroups according to the molecular mechanisms in which the proposed biomarkers are involved (Wilson et al., 2023): pathological protein aggregation; altered energy homeostasis; aberrant proteostasis; inflammation; DNA or RNA defects; synaptic and neuronal network dysfunction biomarkers and other biomarkers (for biomarkers not classifiable according to the new classification of neurodegenerative biomarkers). No study quality threshold was set and all studies were included to further discuss the potential improvement of outcomes or methodology in future studies.

Results

In total, 289 articles published until September 2023 were identified through database searches. A total of 77 studies were removed as duplicates. Out of the remaining 212 studies, 178 were also excluded for not meeting eligibility criteria. Finally, 34 studies were reviewed for data collection

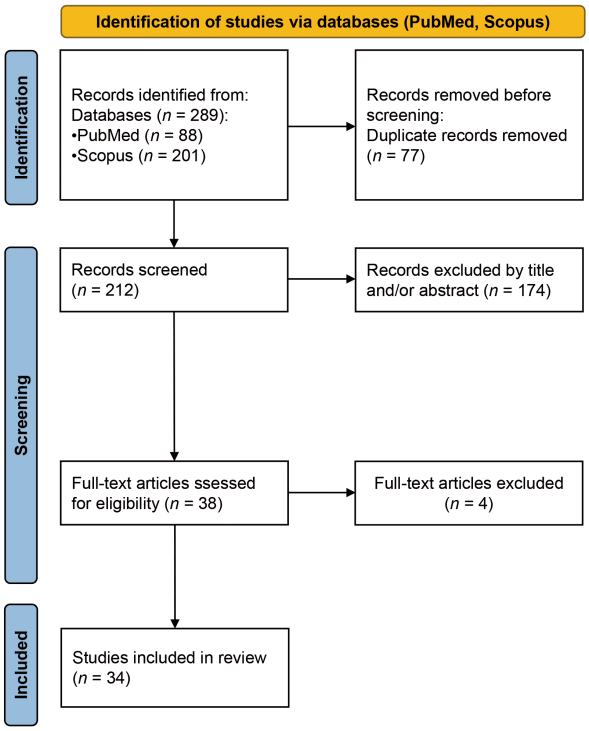

Figure 2 | **PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) diagram for systematic literature reviews.**

(**Figure 2**). Findings, methodologies, and accuracy of each study included in the systematic review are summarised in **Additional Table 1**. As listed below, studies were classified into different categories according to the new classification of neurodegenerative biomarkers (Wilson et al., 2023) and the final subgroup categorization included: 19 studies assessing pathological protein aggregation biomarkers (alpha-synuclein, tau amyloid-β species); 6 studies assessing altered energy homeostasis biomarkers (DJ, heme-oxygenase-1, metabolites); 1 study assessing aberrant proteostasis biomarkers (MAPLC-3beta); 3 studies assessing inflammation biomarkers (cortisol, TNF-alpha); 4 studies assessing DNA or RNA defects biomarkers (DNA methylation, miRNA); 1 study for synaptic and neuronal network dysfunction biomarkers (acetylcholinesterase activity) and 3 studies for other biomarkers (Raman spectra peak, proteome, caffeine). The most reliable and reproducible biomarker findings have been summarized in **Additional Table 2**.

Pathological protein aggregation biomarkers (alpha-synuclein species, tau, and beta-amyloid proteins)

Alpha-synuclein species

In the model of α-syn aggregation, soluble α-syn oligomers are the first aggregates forming in degenerating neurons. Subsequently, oligomers aggregate in fibrils which acquire a beta-sheet conformation and become partially or insoluble. The fibrils in turn contribute, together with lipids and damaged intracellular organelles, in forming Lewy bodies and Lewy neurites (Shahamoradian et al., 2019). Lewy aggregates contain amyloid fibrils of full-length monomeric α-syn- named "native α-syn" - and post-translationally modified forms of α-syn, including phosphorylated α-syn, truncated α-syn, and nitrated α-syn. The portion of phosphorylated α-syn at serine 129 (Ser-129) represents ~90% of α-syn deposited in Lewy bodies, and the effect of phosphorylation seems to accelerate fibrils formation (Fujiwara et al., 2002; Anderson et al., 2006; Paleologou et al., 2008). Excessive nitrative modifications of α-syn, derived from the reaction with nitrating agents, appear to accelerate α-syn aggregation (Souza et al., 2000; Paxinou et al., 2001). α-Syn species investigated in saliva included: oligomeric α-syn, total α-syn, phosphorylated α-syn, and monomeric – "native" α-syn. All the studies performing salivary oligomeric α-syn found significantly increased levels of PD in comparison to HC (Vivacqua et al., 2016; Kang et al., 2016b; Cao et al., 2019; Vivacqua et al., 2019; Shaheen et al., 2020; De Bartolo et al., 2022; Angius et al., 2023). Conversely, conflicting results have been obtained regarding salivary total α-syn levels, with studies showing no difference between PD and HC (Kang et al., 2016a; Cao et al., 2019; Chahine et al., 2020; De Bartolo et al., 2022; Angius et al., 2023), and other studies reporting reduced levels in PD in comparison to HC (Vivacqua et al., 2016, 2019; Shaheen et al., 2020; Sabaei et al., 2023) and in comparison to patients affected by progressive supranuclear palsy (Vivacqua et al., 2019), as well as in multiple system atrophy (MSA) in comparison to PD patients (Cao et al., 2020). Only one recent study reported increased salivary α-syn total levels pertaining to small extracellular vesicles in PD *vs.* HC (P = 0.0093; Rastogi et al., 2023). By calulating the ratio between oligomeric and total α-syn levels, six studies reported increased oligomeric/total α-syn ratios (Vivacqua et al., 2016; 2019; Kang et al., 2016; Cao et al., 2019; Shaheen et al., 2020; Angius et al., 2023).

Conflicting results have been obtained for phosphorylated α-syn in the saliva of PD patients, with studies (Cao et al.; 2019; Angius et al., 2023) finding no differences in phosphorylated α-syn levels and the ratio of phosphorylated α-syn/total α-syn, between PD and HC (Cao et al., 2019), while others (Rani et al., 2019; Rastogi et al., 2023) reporting increased levels of phosphorylated α-syn and an increased ratio phosphorylated α-syn/total α-syn in PD patients in comparison to HC.

Other post-translationally modified forms of α-syn have been investigated in saliva only in one study (Fernández-Espejo et al., 2021), which did not find any differences in PD *vs*. HC. The study inferred to explore the presence of nitrated forms of α-syn in saliva through the assessment of 3-nitrotyrosine proteins, but no differences were found between PD and HC (Fernández-Espejo et al., 2021).

From a methodological point of view, most studies used sandwich ELISA to assess the various forms of α-syn in saliva (Al-Nimer et al., 2014; Vivacqua et al., 2016, 2019; Goldman et al., 2018; Chahine et al., 2020; Shaheen et al., 2020; Fernandez-Espajo et al., 2021; Angius et al., 2023). One recent study performed a competitive ELISA (De Bartolo et al., 2022) showing a superior detection performance for oligomeric α-syn (AUC = 0.99). Optimal diagnostic accuracy has been reported using a different and promising approach, based on neural derived extracellular vesicles extracted from saliva, which have reported high sensitivity and specificity for oligomeric α-syn (sensitivity = 92%, specificity = 86%, AUC: 0.941; Cao et al., 2019) and for total α-syn (sensitivity =

88.24%; specificity = 75%; AUC = 0.8137; Rastogi et al., 2023) in distinguishing PD patients from HC. Recently, aggregation assays based on seeding-competent α-syn species detected in saliva through Real-Time Quaking Induced Conversion (RT-QuIC) assays, have been used in two studies, with optimal accuracy in distinguishing *de novo* PD patients from HC (sensitivity of 83.78%, specificity of 82.61%, AUC = 0.84; Vivacqua et al. 2023) and PD patients in different disease stage from HC (sensitivity = 76%, specificity = 94.4%, AUC = 0.91; Luan et al. 2022). Moreover, in comparison to MSA patients – similarly discriminated by RT-QuIC with respect to HC (sensitivity = 61.1%, specificity 94.4%, AUC = 0.81) – PD patients displayed a significantly shorter lag-phase (i.e. time to threshold) than MSA patients ($P < 0.001$), suggesting that the RT-QuIC lag-phase could be a promising parameter for discriminating different synucleinopathies (Luan et al., 2022).

Correlation with disease severity and/or duration emerged in a few studies (Kang et al., 2016; Shaheen et al., 2020; Vivacqua et al., 2016, 2023). Intriguingly, Kang et al. (2016) found that the oligomer α-syn/total α-syn ratio decreased significantly in Hoehn & Yahr (H&Y) stage I ($P = 0.001$) and increased in H&Y stages II–IV ($P = 0.037$, $P = 0.002$, $P = 0.000$, respectively), with the highest ratio of oligomeric α-syn occurring in the late PD stages. Vivacqua et al. (2016) found that total α-syn levels correlated positively with disease duration ($P = 0.02$), H&Y ($P = 0.02$), Unified Parkinson's Disease Rating Scale (UPDRS) total score part 1–4 ($P = 0.05$), Levodopa Equivalent Daily Dose ($P < 0.01$) and negatively with Montreal Cognitive Assessment ($P = 0.02$) and Frontal Assessment Battery ($P = 0.01$) scores. Shaheen et al. (2020) found a significant correlation between oligomeric α-syn levels and disease duration ($P = 0.03$), and higher oligomeric α-syn levels in PD patients with bradykinetic-rigid dominant phenotype in comparison to tremor dominant phenotype ($P = 0.03$). Only one study, conducted in a sample of only 15 PD patients, described a negative correlation between total α-syn and UPDRS-III score and between phosphorylated α-syn and the Non-Motor Symptoms Scale score (Angius et al., 2023). Interestingly, in exploiting correlations between seeding-competent a-syn oligomers and clinical features in *de novo* PD patients, Vivacqua et al. (2023) detected a significant positive correlation between the summed UPDRS part III and Non-Motor Symptoms Scale score and different RT-QuIC kinetic parameters, indicating that increased disease severity is significantly associated with a greater response in the salivary α-syn RT-QuIC assay.

Tau proteins

Tau is a cytosolic protein and its phosphorylation at residues outside of the microtubule-binding domain has been observed to disrupt the microtubule-dependent axonal transport and perturbing the integrity of neuronal cytoskeleton (Noble et al., 2013). Furthermore, *in vivo* and animal studies suggest that interactions between α-syn and tau can promote their fibrillization and drive the formation of pathological inclusions, thus leading to neurodegeneration (Giasson et al., 2003; Lee et al., 2004). Only two studies investigated tau levels in salivary samples in PD (De Bartolo et al., 2022; Sabaei et al., 2023). Sabaei et al. (2023) found phosphorylated-tau salivary levels not significantly different in PD compared to the control group ($P = 0.104$), reporting a poor diagnostic accuracy (AUC = 0.64). De Bartolo et al. (2022) found increased levels of total tau in *de novo* PD in comparison with HC ($P < 0.00001$), but no significant difference for phosphorylated 199-tau ($P = 0.4442$). A positive correlation between phosphorylated 199-tau and Microtubule-Associated Protein Light chain 3-beta; (MAPLC3beta) ($P < 0.0001$) and a negative correlation between phosphorylated 199-tau and tumor necrosis factor-alpha (TNF-alpha) ($P < 0.0001$) were also detected in the same *de novo* PD cohort. No correlations were observed with motor and cognitive scores, but the PD cohort was at the disease onset and cognitively intact.

Beta-amyloid proteins

The amyloid precursor protein is a glycoprotein involved in maintaining neuronal homeostasis like signaling, neuronal development, and intracellular transport. Amyloid-beta (Aβ) 40 and 42 are peptides derived from proteolytic cleavage of amyloid precursor protein, which cluster together to form insoluble fibrils and amyloid beta plaques, thus leading to neurodegeneration (Sehar et al., 2022). Despite the spreading of α-syn pathology to cortical and limbic regions being considered the strongest correlate of dementia in PD, post-mortem PD studies have relied on the occurrence of Aβ plaques in the striatum of PD patients, considering Aβ striatal pathology a possible substrate for cognitive impairment in PD (Irwin et al., 2013). Only two studies investigated Aβ in saliva in PD *vs*. AD patients and HC (Bermejo-Pareja et al., 2010; Sabaei et al., 2023). Bermejo-Parej et al. (2010) found no differences in salivary concentration of $A\beta_{1-42}$ and $A\beta_{1-40}$ between patients with PD and HC, while Sabaei et al. (2023) assessing a panel comprehending also other biomarkers, found increased levels of $A\beta_{1-42}$ in PD as well as in AD patients in comparison to HC, with a suboptimal diagnostic accuracy in PD group (AUC = 0.77). Correlations with cognitive clinical status in the PD group were not performed in both studies, so it remains unclear whether the lack of significant difference in salivary levels of Aβ between PD patients and HC may rely on the absence of cognitive impairment in the PD group.

Altered energy homeostasis biomarkers

DJ-1 is a multifunctional protein involved in many processes, including regulation of apoptosis and pro-survival signaling, autophagy, inflammatory responses, and protection against oxidative stress (Waak et al., 2009; Oh and Mouradian, 2017). DJ-1 is encoded by the *Park7* gene, and its mutation has been associated with early PD onset (Mencke et al., 2021). Several studies suggest that DJ-1 dysfunction could contribute to PD pathogenesis by inducing oxidative stress and influencing cellular metabolism (Mencke et al., 2021). Despite this body of evidence, only a few studies investigated salivary DJ-1 levels in PD (Devic et al., 2011; Kang et al., 2014; Masters et al., 2015), with a significant difference between PD patients and HC reported only in 1 out of the 3 studies ($P = 0.001$), but it did not survive after adjustment for total protein concentration (Masters et al., 2015). Similarly, in a larger PD cohort (Kang et al., 2014) no differences emerged between PD and HC ($P = 0.72$), although salivary DJ-1 levels in advanced PD patients

(H&Y IV) were significantly higher than in patients in the early-intermediate stage (H&Y I–III) (all $P < 0.01$), suggesting that salivary DJ-1 might be a potential biomarker for monitoring disease progression. The same study showed that salivary DJ-1 levels correlated with nigrostriatal degeneration observed in DAT-SCAN ($P = 0.026$).

In vitro experiments (Tavitian et al., 2020), animal models (Song et al., 2017), and post-mortem PD studies (Schipper et al., 1998) have shown that up-regulation of heme-oxygenase 1 results in increased oxidative stress, excessive iron levels, mitochondrial dysfunction, and macroautophagy in the dopaminergic neurons of the substantia nigra. Despite only two studies investigating salivary heme-oxygenase 1 levels, suboptimal diagnostic accuracy was reached in discriminating PD from HC (AUC = 0.76) (Song et al., 2018) and a good diagnostic accuracy in discriminating PD from other neurodegenerative disorders (patients with Alzheimer's disease and mild cognitive impairment) (AUC = 0.87), from non-degenerative neurological patients (AUC = 0.88) and non-neurological patients (AUC = 0.86) (Galindez et al., 2021).

An NMR-based salivary metabolomic study, found increased levels of phenylalanine, tyrosine, histidine, glycine, acetoacetate, taurine, TMAO, GABA, N-acetylglutamate, acetoin, acetate, alanine, fucose, propionate, isoleucine, and valine in the saliva of PD in comparison to HC (all $P < 0.05$), with AUC ranging from 0.67 to 0.72 (Kumari et al., 2020). The authors also reported correlations for some metabolites with disease duration (propionate and acetoin), Levodopa Equivalent Daily Doses (glycine, taurine, TMAO, isoleucine, and valine), and H&Y stage (butyrate). These metabolites reflect metabolic variations in the production of ketone bodies, mitochondrial respiratory chain, oxidative stress, metabolism of neurotransmitters, and gut microbiota-related metabolites, suggesting that these metabolic pathways may be involved in PD pathogenic mechanisms, also towards the gut-to-brain axis.

Aberrant proteostasis biomarkers

The microtubule-associated protein light chain 3 beta (MAPLC3 beta) is the main factor responsible for the formation of auto-phagolysosomes and the initiation of autophagy. MAPLC3 beta has been considered so far the gold standard for monitoring autophagy in different tissues (Klionsky et al., 2021). Impairment of the autophagy-lysosomal pathway, seems to contribute to the accumulation of aggregated forms of α-syn in different brain areas (Moors et al., 2016), and impaired autophagy has been detected at several levels in PD (Papagiannakis et al., 2015; Miki et al., 2018; Youn et al., 2018). One study reported significantly higher levels of salivary MAP-LC3 beta in *de novo* PD patients in comparison to HC ($P < 0.00001$), showing optimal diagnostic accuracy (AUC = 0.9236) (De Bartolo et al., 2022). Furthermore, MAPLC3 beta salivary levels correlated with Non-Motor Symptoms Scale ($P < 0.001$; De Bartolo et al., 2022).

Inflammation biomarkers

Upregulation of the adrenal pituitary hypothalamus axis, with a consequent increase in cortisol release, has been demonstrated in PD (Ibrahimagic et al., 2016). Despite cortisol not being a disease-specific biomarker, its increase may influence the inflammatory response and contribute to the pathogenesis of PD (Luthra et al., 2022) and some clinical manifestations of PD, including neuropsychiatric symptoms (Soares et al., 2019; van Wamelen et al., 2020). Higher cortisol levels have been proven to induce dopaminergic neurodegeneration (Kim et al., 2021). Increased salivary cortisol levels have been reported in PD in 2 studies without ($P = 0.019$; Djamshidian et al., 2011) and with ($P = 0.03$; Costa et al., 2019) association with psychiatric symptoms.

TNF-alpha is a proinflammatory cytokine that seems to induce a pro-inflammatory phenotype in microglia (Smith et al., 2012; Brás et al., 2020), contributing to the degeneration of dopaminergic neurons (Allan and Rothwell, 2001). TNF-alpha was investigated in salivary samples of PD patients only in 1 study, in association with other biomarkers, and was found significantly higher in *de novo* PD patients in comparison to HC ($P < 0.00001$), although its diagnostic accuracy was not optimal (AUC = 0.66; De Bartolo et al., 2022). In the same study, salivary levels of TNF-α negatively correlated with those of salivary phosphorylated 199-tau, suggesting interplay between tau pathology and inflammation in *de novo* PD.

DNA and RNA defect biomarkers

A saliva-based DNA methylation study reported five significant PD-associated CpGs ($P < 10^{-7}$), two of which were located in the H-ferritin genes encoding for a protein involved in iron uptake and oxidation in the brain and the other one implicated in immune-associated module (Chuang et al., 2017).

Specific miRNAs, small non-coding RNA molecules, have been shown to regulate PD-related gene expression and intervene in several pathways including apoptosis, neuroinflammation, mitochondrial dysfunction, and proteasomal degradation in PD (Goh et al., 2019). Furthermore, it has been observed that some miRNAs can be directly involved in α-syn aggregation (Recasens et al., 2016). miRNA-874 and miRNA-145-3p (the specific miRNAs that seem to regulate the expression of DJ-1) have been found highly expressed in the saliva of PD patients in comparison to HC, with AUC ranging from 0.70 to 0.72 (Chen et al., 2020). While miRNA-153 and miRNA-223 (probably involved in the regulation of α-syn expression) were found to be under-expressed in the saliva of PD patients in comparison to HC ($P = 0.01$ and $P = 0.02$, respectively), with an AUC of 0.77 (miRNA-223) and 0.79 (miRNA-153). However, the ratio between miRNA-153 and miRNA-223 and both oligomeric and total α-syn revealed only an increased oligomeric α-syn/miRNA-153 ratio between PD patients and HC (Cressatti et al., 2020). Jiang et al. (2021) tested, in a validation phase, eight different candidate miRNAs and found downregulated salivary expression of miRNA-29a-3p ($P = 0.004$) and miRNA-29c-3p ($P = 0.027$) (miRNAs involved in regulating apoptosis and cell death) and upregulated expression of miRNA-6756-5p (with unclear role) ($P = 0.032$) in PD patients in comparison to HC. Then, they tested the diagnostic accuracy of miRNA-29a-3p, miRNA-29c-3p, and miRNA-6756-5p in discriminating PD

from MSA and essential tremor patients, finding an optimal accuracy only for miRNA-29a-3p (AUC = 0.89) in distinguishing PD from MSA patients.

Synaptic and neuronal network dysfunction biomarkers

Salivary AChE activity has been proposed as a potential biomarker for central cholinergic dysfunction and parasympathetic denervation (Sayer et al., 2004). One study, investing salivary AChE activity in PD, found increased activity in PD patients in comparison to HC ($P < 0.001$), as well as increased total protein concentration ($P = 0.002$) and increased ratio of AChE activity/total protein levels ($P = 0.04$) (Fedorova et al., 2015). Interestingly, the correlation was seen between color vision test performance and AChE activity ($P = 0.04$).

Other biomarkers

One study performed Raman spectroscopy in saliva samples of PD, AD, and HC. Using machine learning and principal component analysis, it evaluated which prominent Raman peaks were able to cluster subjects according to the diagnosis (Carlomagno et al., 2021). Raman spectra analysis showed differences regarding peaks related to proteins (signals from specific amino acids and amide bands), nucleic acids, and glycoproteins/saccharides able to discriminate PD from AD patients and HC, with an optimal diagnostic accuracy (AUC = 0.98; Carlomagno et al., 2021). More specifically, in the subtraction spectrum between PD and AD patients, the most prominent peaks in PD were related to proteins and phosphatidylinositol. A correlation was found between Raman peaks and disease severity assessed with UPDRS part III and H&Y in PD, suggesting a close relationship between the salivary biochemical content and the clinical state of the patient. Moreover, comparing the salivary proteome of PD patients and HC, Figura et al. (2021) found lower expression of S100-A16, ARP2/3, and VPS4B proteins (respectively involved in adipose tissue formation, DNA repair, and exosome formation) in PD in comparison to HC, but with unsatisfactory diagnostic accuracy (AUC ranging from 0.4 to 0.7).

Robust previous evidence suggests that caffeine is a protective factor in PD, probably due to its action as an antagonist of adenosine type-2A receptors, which are involved in neuroprotection of dopaminergic neurons (Xu et al., 2010; Postuma et al., 2012; Ascherio and Schwarzschild, 2016; Belvisi et al., 2020). However, salivary caffeine levels did not show any significant differences in de novo/early PD patients in comparison to HC ($P = 0.15$), but decreased salivary caffeine levels were found in moderate/advanced PD *vs*. HC ($P = 0.009$), suggesting that salivary caffeine cannot be used as a diagnostic biomarker but more properly as a biomarker of disease progression in PD (Leodori et al., 2021).

Discussion

In this review, we have first depicted the biological basis for looking at saliva in the search for neurodegenerative biomarkers and we have then reported the results achieved so far in saliva samples from studies investigating biomarkers in PD.

Non-invasive and pain-free collection procedures make saliva one of the most accessible and feasible sources for biomarker detection in PD, especially considering its potential application for the prevention of longitudinal studies in large cohorts of healthy individuals.

From our systematic revision of the literature, α-syn has been the most investigated PD biomarker in saliva so far. Among various α-syn species investigated, salivary oligomeric α-syn showed the most consistent findings. All the studies concordantly reported increased levels of salivary oligomeric α-syn in PD patients, while there are still conflicting results regarding the levels of total α-syn and phosphorylated α-syn. Few studies have reported an increased oligomeric α-syn/total α-syn ratio (Kang et al., 2016; Vivacqua et al., 2016; 2019; Cao et al., 2019; Shaheen et al., 2020).

Determination of α-syn species pertaining to neural derived extracellular vesicles has reported an optimal diagnostic accuracy for salivary oligomeric α-syn (AUC = 0.94; Cao et al., 2019) and satisfactory accuracy for total α-syn (AUC = 0.81; Rastogi et al., 2023), with a better performance in comparison to the one achieved from extracellular vesicles isolated from serum (Agliardi et al., 2021, 2022). In the same direction, improvements in immunoenzymatic assays allowed to encompass detection limits intrinsic to α-syn oligomers molecular variability, with competitive ELISA detection of α-syn oligomers, showing an optimal diagnostic accuracy (AUC = 0.99) in discriminating *de novo* PD patients from HC (De Bartolo et al., 2022), also considering low costs and feasible standardization of ELISA assays in different laboratories. The recent diffusion of seeding-aggregation assays for α-syn (including RT-QuIC), further overcame detection issues relative to epitope recognition on α-syn oligomers, exploring directly the presence of pathologic seeding-competent α-syn species in biological fluids. RT-QuIC assays performed on saliva samples have been shown to discriminate PD from HC (Luan et al., 2022; Vivacqua et al., 2023), even in the early stage of the disease (Vivacqua et al., 2023) with sensitivity and specificity values near to those reported in studies employing the same methodology on skin biopsies and CSF samples (Bellomo et al., 2022; Grossauer et al., 2023). This is highlighted in a recent systematic review and meta analysis in which comparable pooled sensitivity and specificity are reported for saliva and CSF, assessed through α-syn RT-QuIC (Zheng et al., 2023). Although a limited number of laboratories can perform RT-QuIC assays, and the assay's methodology varies widely between research groups, it is worth noting that kinetic parameters of salivary α-syn RT-QuIC correlate with severity of motor and non-motor symptoms in *de novo* PD patients (Vivacqua et al., 2023), encouraging further studies on larger cohorts. Comparative studies between saliva and other biofluids/peripheral tissues in PD are highly encouraged, since only 2 studies (Goldman et al., 2018; Chahine et al., 2020) have been realized with this purpose, but they did not find a sufficient diagnostic accuracy for salivary biomarkers. In these studies only total α-syn has been measured, not considering oligomeric α-syn which is likely the most reproducible and reliable salivary biomarker in PD (Goldman et al., 2018; Chahine et al., 2020).

Despite α-syn being the signature biomarker of PD, its levels do not seem to vary among different subtypes (Goldman et al., 2018; Chahine et al., 2020; De Bartolo et al., 2022), thus suggesting that the contribution of other molecular pathways may play a more relevant role in determining clinical and pathological heterogeneity observed in PD. Several pieces of evidence from animal models, post mortem, and living PD patients suggest the involvement of microglia activation, autophagy/proteasome impairment, DNA and RNA defects, and synaptic and neuronal network dysfunction in the pathogenetic mechanisms underlying PD (Poewe et al., 2017; Wilson et al., 2023). In this direction, a few studies in saliva have investigated parallel biomarkers pertaining to different potential pathogenetic pathways, reporting promising results. Significantly altered levels of salivary total tau, phosphorylated tau, and $A\beta_{1-42}$ (De Bartolo et al., 2022; Sabaei et al., 2023) were reported in PD patients, despite the lack of clinical correlations. The search of tau and amyloid beta species in saliva, deserves to be implemented in larger PD population studies with and without cognitive impairment, to verify whether they can predict the transaction to dementia in PD. Altered energy homeostasis biomarkers; aberrant proteostasis biomarkers; inflammation biomarkers; DNA/RNA defects biomarkers; and synaptic and neuronal network dysfunction biomarkers have been explored in separate studies. Although the studies on these biomarkers in saliva were not replicated in PD, making it difficult to address their reproducibility, the reported results are also encouraging and support their future employment in larger cohorts. Notwithstanding, since these biomarkers are involved in pathogenic mechanisms underlying also other neurodegenerative disorders rather than PD (Wilson et al., 2023), it is crucial to combine their assessment with signature pathogenic proteins (i.e. α-syn, tau, amyloid-β), and to define whether these pathogenetic pathways play a role in determining the molecular variance among PD patients.

The same complexity of salivary secretion, which results advantageous for a comprehensive biomarker detection, represents the principal biological limitation for reliability and reproducibility of salivary-based biomarker studies. Salivary secretion rate presents circadian and circannual variations, leading to intra and inter-individual changes of the salivary secretion and composition, which are exacerbated in the PD population, where hypersialorrhea or hyposialorrhea occur in relation to the stage of the disease and the pharmacological therapy (Tumilasci et al., 2006; Cersósimo et al., 2009). Variations in pH, total protein concentration, electrolyte concentration, or concentrations of specific proteins and metabolites can potentially affect the reliability and the reproducibility of biomarkers measurements in saliva (Vivacqua et al., 2018; Song et al., 2023). The presence of proteolytic and lipolytic enzymes is strongly relevant in saliva and requests a standardized treatment with protease and lipase inhibitors before applying any biological measurement (Vivacqua et al., 2018). DNA and RNA detections in saliva might be contaminated by the presence of prokaryotic genetic material, which may lead to possible failure of genomic and transcriptomic analyses (Abraham et al., 2012). This implies the preliminary design of human-specific RNA and DNA libraries to employ in saliva. The presence of different mucins proteins and their structural modifications in relation to salivary hydration represents a biological limitation in the detection of pathogenic proteins. Expansion of mucins with high hydration can increase the matrix effect of saliva (Tabak et al., 1982), hampering an efficient antibody binding in ELISA. Sequestering of seeding competent aggregates into the mucins matrix, can prevent the nucleation phase of aggregation assays (Davenport et al., 2018). Moreover, the fibrillar organization of mucins, similar to the amyloid species of pathogenic proteins, can affect the specificity of their detection in saliva (Kesimer et al., 2010). Subjects affected by diabetes mellitus, autoimmune diseases, systemic inflammatory diseases, neoplasms, and salivary gland/oral cavity pathologies may not be eligible for salivary tests. Furthermore, salivary collection should be performed after the overnight fasting, to avoid the interference of eating, drinking, smoking, or alcohol consumption (Vivacqua et al., 2018; Zürcher and Humpel, 2023). After collection, the salivary samples need to be speedily treated with protease/lipase inhibitors and stored at –80°C before the biological analyses (Vivacqua et al., 2018; Zürcher and Humpel, 2023). Further studies are also needed to detect whether differences in salivary biomarkers occur, in relation to the circadian and the circannual rhythms.

On the other hand, the employment of saliva discloses many promising perspectives. The release of neuronal-derived vesicles in saliva, due to the close relation of the autonomic fibers with salivary-secreting cells and with cells of the salivary ducts, makes saliva a possible source of biomarkers with direct nervous derivation (**Figure 1B**). Exocytosis of salivary exocrine vesicles resamples the trafficking of synaptic vesicles (Fujita-Yoshigaki et al., 1998; Wang et al., 2007), representing a potential peripheral source to investigate synaptic dysfunction (e.g., rearrangement of the SNARE complex; **Figure 1E**) characterizing the early PD (Wegrzynowicz et al., 2019; Garcia-Reitböck et al., 2010). Abundance of mitochondria in the cells lining the striate ducts of the salivary glands is a potential exchange surface between products of the mitochondrial metabolism and saliva, which could be employed to investigate biomarkers of oxidative stress or altered energy homeostasis (**Figure 1D**). Finally, the intricate molecular composition of saliva, including structural proteins, enzymes, lipids, a variety of metabolites as well as cell fragments and resident cells, supports the possibility of comprehensive biomarkers studies, towards a multi-omic approach, which constitutes the most promising strategy for stratifying PD patients into molecular subtypes.

In conclusion, saliva emerges as a promising source for detecting biomarkers in PD. Biomarkers tested in saliva have demonstrated diagnostic accuracy often comparable to CSF. However, before salivary-based biomarkers can be adopted in large-scale longitudinal cohorts and multicentric studies, biological challenges associated with the unique molecular features of saliva must be addressed. Crucially, the standardization of sample collection and processing techniques for various molecular assays is essential to ensure the reliability and reproducibility of the results. This step is pivotal for transitioning salivary biomarkers into a real clinical

setting. Given its non-invasive accessibility, saliva should receive greater focus in future research than other biofluids or peripheral tissues requiring more invasive collection procedures.

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Additional files:
Additional file 1: *Open peer review reports 1 and 2.*
Additional Table 1: *Systematic review of studies assessing salivary biomarkers in Parkinson's disease.*
Additional Table 2: *Summary of the main findings in salivary biomarkers studies in Parkinson's disease patients in comparison with healthy controls.*

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Additional Table 2 Summary of the main findings in salivary biomarkers studies in Parkinson's disease patients in comparison with healthy controls

Biomarkers	Main findings	Number of studies
Oligomeric a-syn	Increased	7
Total a-syn	Mostly unchanged	6 out of 11
Oligomeric/total a-syn ratio	Increased	5
Seeding competent a-syn species	Increased	2

a-Syn: Alpha-synuclein