Review

Salivary biomarkers of neurodegenerative and demyelinating diseases and biosensors for their detection

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A B S T R A C T

Salivary analysis is gaining increasing interest as a novel and promising field of research for the diagnosis of neurodegenerative and demyelinating diseases related to aging. The collection of saliva offers several advantages, being noninvasive, stress-free, and repeatable. Moreover, the detection of biomarkers directly in saliva could allow an early diagnosis of the disease, leading to timely treatments. The aim of this manuscript is to highlight the most relevant researchers' findings relatively to salivary biomarkers of neurodegenerative and demyelinating diseases, and to describe innovative and advanced biosensing strategies for the detection of salivary biomarkers. This review is focused on five relevant aging-related neurodegenerative disorders (Alzheimer's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, Huntington's disease, Multiple Sclerosis) and the salivary biomarkers most commonly associated with them. Advanced biosensors enabling molecular diagnostics for the detection of salivary biomarkers are presented, in order to stimulate future research in this direction and pave the way for their clinical application.

1. Introduction

Better living conditions and more efficient healthcare infrastructures are increasingly extending the median lifetime of the European and US population. The median age in Europe is, in fact, expected to increase by almost five years by 2050 (European Union, 2020), while individuals with more than 65 years living in the US are projected to be 88 million by the same year (Alzheimer Association, 2018). As the share of old people among the population increases, the burden associated with age-related diseases is going to increase substantially (Hou et al., 2019). Therefore, it is necessary to rapidly develop innovative therapies and preventive measures to efficiently contrast the aging trend. Among several age-related diseases, neurodegenerative diseases have a great incidence on the population, and can be classified among the most burdening on a lifestyle perspective, accompanied by severe impairments and deficits (Hou et al., 2019).

Neurodegenerative diseases (NDDs) are a miscellaneous group of

disorders, such as Alzheimer's disease (AD), Huntington's disease (HD), Parkinson's disease (PD) and Amyotrophic Lateral Sclerosis (ALS). Neuronal degeneration also occurs in later stages of other diseases, such as Multiple Sclerosis (MS), initially characterized by demyelination but leading over time to progressive neuronal loss (Kovacs, 2017). Several mechanisms, such as mitochondrial dysfunction, excitotoxicity and extracellular aggregation of toxic molecules are responsible for neuronal death, and this can lead to both cognitive and motor impairment (Bossy-Wetzel et al., 2004). An early diagnosis, as well as the prediction of the disease course or the response to treatment, can therefore be fundamental to improve the patient's quality of life. It could prevent the irreversible damage associated with NDDs and MS, such as neuronal loss, and allow the start of an appropriate therapy, which could be more effective at an early stage (Galvin and Sadowsky, 2012). In this context, the detection of biomarkers in biological fluids could represent a useful diagnostic tool and it is important to find an easy, noninvasive, painless and safe method to detect these biomarkers and to make early diagnosis

(Gleerup et al., 2019). Fig. 1 provides a summary of the biofluids that are commonly used for the detection of biomarkers, along with their advantages and disadvantages.

Cerebrospinal fluid (CSF) is considered the gold standard for the detection of biomarkers associated with NDDs or damage of the brain tissue, mainly due to its proximity to the neuronal environment. The use of this biofluid typically ensures having a high accuracy in determining the concentration of the biomarkers, but the sampling procedure is highly invasive, requiring hospitalization (Ferreira et al., 2014; Sancesario and Bernardini, 2019; Swift et al., 2021). In addition, repeated measurements are difficult to achieve for the organization and costs required for the procedure, and the compliance of patients. More recently, other technologies, such as genomics, transcriptomics, proteomics, metabolomics, secretomics etc. have also gained a pivotal role in the diagnosis, relying on noninvasive methods, like the collection of blood, tears, urine, and saliva (Ausó et al., 2020; Hrubešová et al., 2019). Among these, blood is a reproducible, simple and convenient method to detect biomarkers, but it is less accurate than CSF, as well as stressful and moderately invasive (Ausó et al., 2020). Saliva is a biofluid that has been demonstrated being rich in biomarkers (Malon et al., 2014). Several hypotheses have been proposed to justify the presence of these biomarkers: they can pass from the blood via active transport, passive intracellular diffusion, or microfiltration; otherwise, they could be excreted from neuronal axons or be produced in the salivary glands (Gleerup et al., 2019; Reale et al., 2020). The collection of saliva has several advantages: it is noninvasive, stress-free, easy and it can be repeated if needed without any biological cost. Moreover, it requires neither special storage conditions nor clinician training and it is largely available and frequently renewed (Hartmann and Kist, 2018; Sancesario and Bernardini, 2019; Schepici et al., 2020). Saliva can be easily obtained from the burr or through stimulation methods, which allow collecting a larger quantity of sample. Moreover, saliva is easier to handle than blood, it does not clot, it is stable over time and provide little to no risk of cross-contamination or exposure to pathogens (Malon et al.,

2014). Altogether these advantages are fostering saliva as a promising tool for remote monitoring and point of care (POC) diagnostics (Goswami et al., 2015; Javaid et al., 2016). On the other side, it has been proven how the analytes' concentration is typically lower compared to blood and it could be influenced by several factors, such as physiological states, stressors, as well as the diurnal/circadian variations (Gleerup et al., 2019; Reale et al., 2020). As of now, the variability in the methods of collecting, preserving, and analyzing saliva, together with the lack of clear ranges for the diagnosis limit the use of saliva in clinical analysis. Salivary analysis is usually performed in centralized laboratories, with highly specific equipment requiring trained personnel. These cumbersome procedures are linked with the high costs of salivary analysis that further discourage its application in clinical practice. In this context, the use of biochemical sensors that are able to detect analytes in a given body fluid, represents a viable and concrete opportunity to make an early targeted diagnosis and treatment of diseases. Given the limitations of the conventional methods of analysis, the research moved towards biosensors targeting salivary biomarkers in the past decade. Since the term "salivaomics" was coined in 2008 by prof. D.T. Wong at UCLA, many biosensors allowing for rapid and affordable diagnostics have emerged along with several review papers summarizing these efforts. Although many review papers have been published on the topic of salivary biomarkers and biosensors, a manuscript summarizing the most recent findings on biosensors targeting salivary biomarkers for neurodegenerative and demyelinating diseases is still lacking.

Therefore, the aim of this review is twofold: on one hand we wish to provide the reader with an overview of the state of the art on salivary biomarkers for the diagnosis of five NDDs that are commonly associated with an aging population. On the other, the aim is to present a few examples of biosensors that have recently been developed to detect salivary biomarkers related to NDDs, and wearable sensing platforms that are foreseen to facilitate the evaluation of the concentration and circadian rhythm of specific biomarkers. This manuscript is aimed to increase awareness in the field of salivary biomarkers related to age-related NDDs
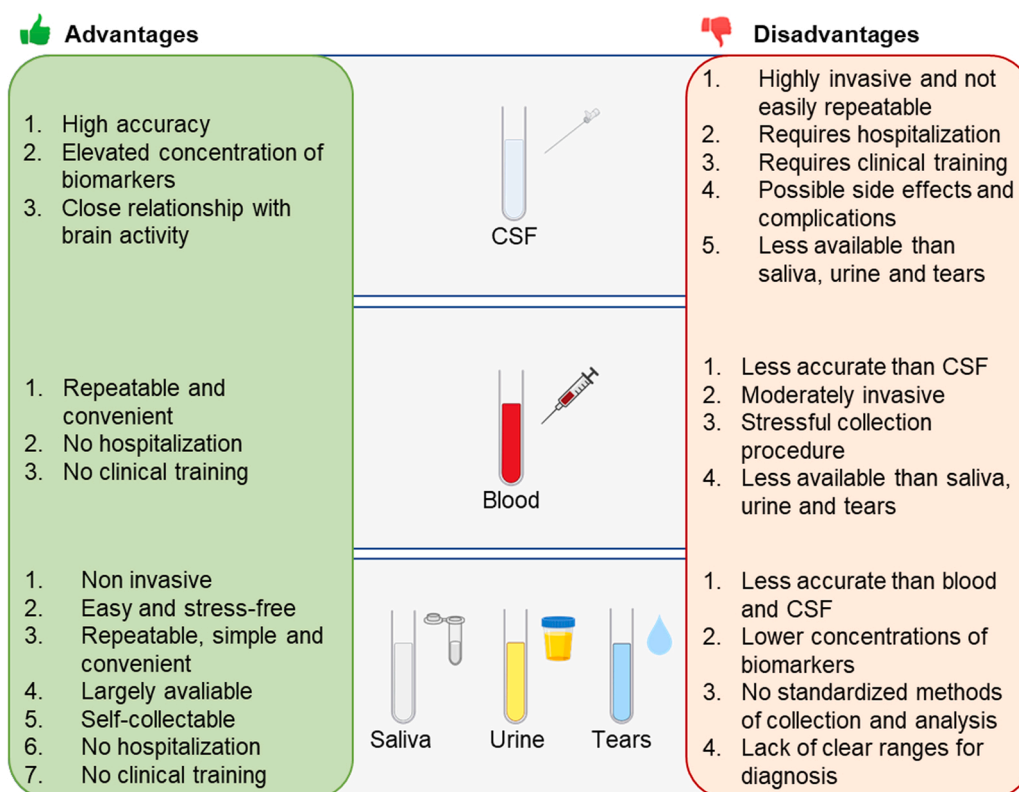

Fig. 1. Advantages and disadvantages of biological fluids used for the detection of clinically relevant biomarkers.

and foster the research efforts regarding the use of innovative biosensors to detect them.

2. Alzheimer's disease

One of the most common NDDs is Alzheimer's disease (AD), which is the most frequent type of dementia, representing 60–70% of cases (World Health Organization, 2017). Its diagnosis, besides clinical and neuropsychological assessment, is currently performed through magnetic resonance imaging (MRI), positron emission tomography (PET) and CSF analysis (mainly in European countries).

However, the presence of conditions that can resemble normal ageing, such as frailty or mild cognitive impairment, may contribute to make diagnosis of dementia difficult (Robinson et al., 2015). Moreover, by the time overt symptoms arise, loss of integrity of neurons has already occurred in many regions, while biochemical changes are believed to occur decades before the onset of symptoms (Bateman et al., 2012; Villemagne et al., 2013). In this context, several studies focused on the detection of salivary biomarkers in hopes of validate them as useful alternatives for an early diagnosis of dementia (Bermejo-Pareja et al., 2010; Kim et al., 2014a; Lee et al., 2017). However, it is known that peripheral levels of the majority of proteins are much lower than in CSF. Therefore, other Authors proposed to focus on the identification of peripheral biomarkers with a high negative predictive value to be used in the context of primary care setting, i.e. a screening tool not to be intended as diagnostic but as a gatekeeper to further confirmed diagnostic procedures (CSF biomarkers, PET with amyloid tracer) (Hampel et al., 2018).

Concerning pathogenesis of AD, accumulation of amyloid beta peptides ($A\beta$) and formation of neurofibrillary tangles (NFTs), made of phosphorylated tau proteins, are considered the main causes of AD (Fig. 2). More specifically, in pathological conditions β and γ-secretase process APP (Amyloid Precursor Protein), leading to the formation of two peptides, called $A\beta_{1-40}$ and $A\beta_{1-42}$, whose aggregation determines the accumulation of amyloid plaques (Zhang et al., 2012). On the other hand, Tau protein is part of microtubule associated proteins; in AD, phosphorylated form of tau (p-tau) accumulates, leading to intracellular neurofibrillary tangles (NFTs) and, eventually, to neuronal death (Hyman et al., 2005; Bloom, 2014). These proteins, namely $A\beta_{1-40}$, $A\beta_{1-42}$, total tau (t-tau) and phosphorylated tau (p-tau), are the principal biomarkers analyzed in scientific literature for the diagnosis of AD and its early phase, namely Mild Cognitive Impairment (MCI) (Table 1).

However, postmortem studies consistently demonstrate that "pure" pathologies are the exception rather than the rule with neurodegenerative diseases. For example, at autopsy, $A\beta$ and tau are found also in Lewy Body Dementia (LBD) or, conversely, synuclein, typical of LBD, has been observed in AD brain as well (Jellinger, 2008; Sonnen et al., 2010). Also clinically, the differential diagnosis between AD and LBD is challenging, particularly at early stages.

CSF $A\beta_{1-42}$ is already a validated biomarker for AD, where its concentration is known to be decreased in patients with AD compared to healthy controls (Sunderland et al., 2003). Regarding salivary $A\beta_{1-42}$ levels for the detection of AD, conflicting results are reported in literature. In contrast with CSF results, most of the studies show higher levels of its salivary form in AD patients than in healthy controls or patients with Parkinson's disease (Bermejo-Pareja et al., 2010; Kim et al., 2014a; Lee et al., 2017; Sabbagh et al., 2018). This leads to the hypothesis that salivary $A\beta_{1-42}$ is specific for AD and, therefore, it might be an important salivary biomarker for the diagnosis of AD (Bermejo-Pareja et al., 2010; Lee et al., 2017). Notably, levels in MCI are intermediate between AD and non-demented subjects, suggesting that this modification occurs early during the pathogenesis of the disease (Kim et al., 2014a). Still, there are not clear ranges for the diagnosis and even the correlation with the severity and progression of the disease is uncertain (Bermejo-Pareja et al., 2010; Kim et al., 2014a). Results from Lee at al. might indicate the use of salivary biomarkers not only in the diagnosis but also in the prediction of risk of the disease, which seems higher in subjects with a family history of AD (Lee et al., 2017).

However, other researchers either failed to detect the presence of $A\beta_{1-42}$ in saliva (Lau et al., 2015; Shi et al., 2011) or reported a decrease in salivary concentrations of this biomarker, mirroring its trend in CSF (Tvarijonaviciute et al., 2020). The conflicting results emerging from the analyzed studies could be attributed to different methods used to collect, store, process and analyze saliva samples (i.e., ELISA kit, antibody based magnetic nanoparticle immunoassay or other specific kits). Moreover, they could be caused by differences in the screened population and inclusion criteria (Lee et al., 2017; Tvarijonaviciute et al., 2020). On the contrary, salivary concentrations of $A\beta_{1-40}$ did not significantly change between AD patients and controls (Bermejo-Pareja et al., 2010; Kim et al., 2014a). Another biomarker which is widely studied is the salivary tau protein. In CSF both total tau (t-tau) and phosphorylated tau (p-tau) are increased in AD patients compared to healthy controls and, particularly when combined with analysis of $A\beta_{1-42}$, they can be considered an accurate diagnostic tool for AD (Blennow et al., 2001; Blennow and Zetterberg, 2013; Pekeles et al., 2019).

As concerns saliva, most of the studies reported an increase in salivary concentration of p-tau, and consequently of p-tau/t-tau ratio in patients with AD (Lau et al., 2015; Pekeles et al., 2019; Shi et al., 2011). On the other hand, t-tau seems to decrease or remain the same in patients compared to healthy controls (Ashton et al., 2018; Lau et al., 2015; Shi et al., 2011). Nonetheless, salivary concentrations of t-tau and p-tau are subjected to variability and, as reported by Pekeles et al., the wide discrepancy in tau phosphorylation levels in AD patients questions its validity as a diagnostic biomarker (Pekeles et al., 2019). Moreover, p-tau/t-tau ratio did not result significantly correlated with the CSF value (Pekeles et al., 2019).
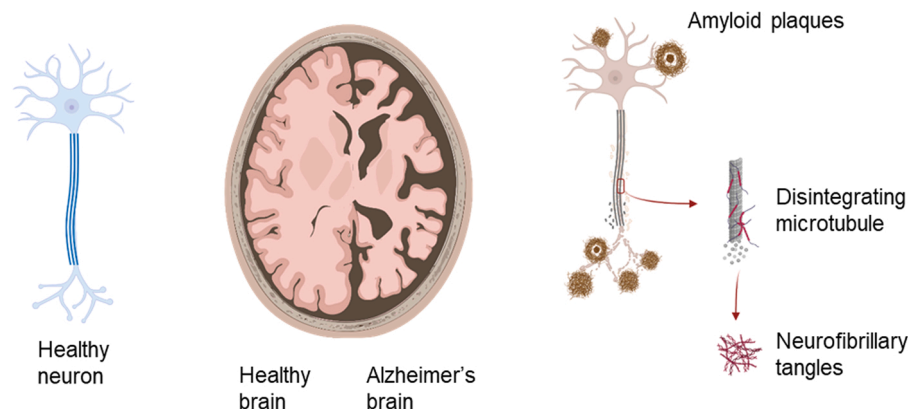

Healthy neuron

Healthy brain   Alzheimer's brain

Amyloid plaques

Disintegrating microtubule

Neurofibrillary tangles

Fig. 2. Alzheimer's disease and its pathogenesis.

Table 1

Salivary levels of biomarkers in Alzheimer's disease.

Biomarker	AD patients	MCI subjects	Non demented subjects	Reference
$A\beta_{1-42}$	6.81 ± 20.04 pg ml^{-1} Mild:7.67 ± 16.25 pg ml^{-1} Moderate:11.70 ± 34.76 pg ml^{-1} Severe:3.03 ± 3.49 pg ml^{-1}		2.89 ± 4.96 pg ml^{-1c} 3.66 ± 4.21 pg ml^{-1d}	(Bermejo-Pareja et al., 2010)
	Higher values compared to HC[a]	Lower values compared to severe AD[a]	Lower values[a,c]	(Kim et al., 2014a)
	59.07 ± 6.33 pg ml^{-1}		22.06 ± 0.41 pg ml$^{-1c,d}$	(Lee et al., 2017)
	51.7 ± 1.6 pg ml^{-1}		21.1 ± 0.3 pg ml^{-1c}	(Sabbagh et al., 2018)
	Not detectable		Not detectable	(Shi et al., 2011)
	Not detectable		Not detectable	(Lau et al., 2015)
	3.15 ± 0.72 pg ml^{-1}		3.57 ± 0.93 pg ml^{-1c}	(Tvarijonaviciute et al., 2020)
$A\beta_{1-40}$	22.3 ± 4.88 pg ml^{-1} Mild:21.87 ± 5.7 pg ml^{-1} Moderate:21.5 ± 4.17 pg ml^{-1} Severe:23.92 ± 4.55 pg ml^{-1}		20.82 ± 5.55 pg ml^{-1c} 26.41 ± 5.12 pg ml^{-1d}	(Bermejo-Pareja et al., 2010)
	Higher values compared to HC[b]	Lower values compared to severe AD[b]	Lower values[b,c]	(Kim et al., 2014a)
p-TAU	Higher values[b]		Lower values[b,c]	(Shi et al., 2011)
	Higher values[b]		Lower values[b,c,d]	(Lau et al., 2015)
p-TAU/t-TAU ratio	Higher values[a]		Lower values[a,c]	(Shi et al., 2011)
	Higher values[a]		Lower values[a,c]	(Pekeles et al., 2019)
t-TAU	Tendency to lower values		Tendency to higher values[c]	(Shi et al., 2011)
	No statistically significant difference		No statistically significant difference[c,d]	(Lau et al., 2015)
	12.2 ng l^{-1}	9.8 ng l^{-1}	9.6 ng l^{-1c}	(Ashton et al., 2018)
lactoferrin	Lower values[a]	Lower values[a]	Higher values[a]	(Carro et al., 2017)
	4.78 ± 1.11 µg ml$^{-1\ g}$		Sub-cohort: 10.24 ± 1.96 µg ml$^{-1c,g}$	
	3.6 ± 1.5 µg ml^{-1}	3.8 ± 2 µg ml^{-1}	7.7 ± 2.4 µg ml^{-1c} 9.7 ± 2.9 µg ml$^{-1\ f}$ 9.8 ± 5.4 µg ml^{-1c}	(Sánchez et al., 2020)
AChE activity	13.70		17.30[c]	(Bakhtiari et al., 2017)

AD: Alzheimer's disease; PD: Parkinson's disease; HC: healthy control; MCI: mild cognitive impairment; FTD: frontotemporal dementia; $A\beta_{1-42}$: β_{1-42} amyloid peptide; $A\beta_{1-40}$: β_{1-40} amyloid peptide; p-TAU: phosphorylated-TAU; t-TAU: total-TAU; AChE: acetylcholinesterase.

[f] FTD subjects.
[a] Statistically significant.
[b] Not statistically significant.
[c] HC subjects.
[d] PD subjects.

Other biomarkers are currently being evaluated for the potential use in the diagnosis of AD. Among these, salivary lactoferrin proved to be a useful tool for early diagnosis of AD, resulting decreased in patients compared to healthy controls and subjects with frontotemporal dementia (Carro et al., 2017; Sánchez et al., 2020). Another example is Acetylcholinesterase (AChE), which catalytic activity decreased in patients with AD, particularly in those who did not respond to AChE-I

treatment (Bakhtiari et al., 2017). Nevertheless, AChE levels reflect neuronal damage, thus likely they are not useful to identify cases before major neuronal damage has occurred.

3. Parkinson's disease

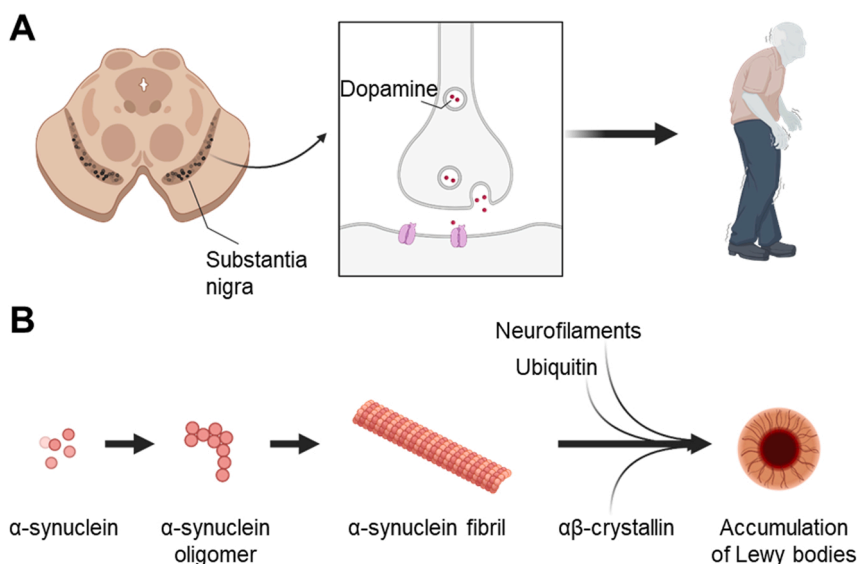Parkinson's disease is a progressive neurodegenerative disorder

Fig. 3. (A) Loss of dopaminergic neurons in the pars compacta of the substantia nigra leads to Parkinson's disease and symptoms (B) Process of Lewy body formation.

Table 2
Salivary levels of biomarkers in Parkinson's disease.

Biomarker	PD patients	Healthy subjects	Reference
total alpha-synuclein	65 ± 52.2 pg ml^{-1}	314.01 ± 435.9 pg ml^{-1c}	(Al-Nimer et al., 2014)
	Lower values[b]	Higher values[b,c]	(Devic et al., 2011)
	5.08 ± 3.01 pg ml^{-1}	31.3 ± 22.4 pg ml^{-1c}	(Vivacqua et al., 2016)
	159.4 ± 61.6 ng ml^{-1}	229.9 ± 64 ng ml^{-1c}	(Shaheen et al., 2020)
	7.104 ± 5.122 pg ml^{-1}	28.444 ± 25.877 pg ml^{-1c}	(Vivacqua et al., 2019)
		29.091 ± 18.677 pg ml^{-1d}	
α-syn$_{olig}$	1.062 ± 0.266 ng ml^{-1}	0.498 ± 0.203 ng ml^{-1c}	(Vivacqua et al., 2016)
	47.8 ± 11.8 ng ml^{-1}	39.2 ± 9.2 ng ml^{-1c}	(Shaheen et al., 2020)
	0.893 ± 1.949 ng ml^{-1}	0.217 ± 0.191 ng ml^{-1c}	(Vivacqua et al., 2019)
	10.39 ± 1.46 pg ng^{-1}	1.37 ± 0.24 pg ng^{-1c}	(Cao et al., 2019)
α-syn$_{olig}$/ α-syn$_{tot}$ ratio	0.174 ± 0.044	0.065 ± 0.027^c	(Vivacqua et al., 2016)
	0.35 ± 0.18 ng ml^{-1}	0.19 ± 0.08 ng ml^{-1c}	(Shaheen et al., 2020)
	0.235 ± 0.793	0.0126 ± 0.0079^c	(Vivacqua et al., 2019)
	1.70 ± 0.52 pg ng^{-1}	0.67 ± 0.26 pg ng^{-1c}	(Cao et al., 2019)
DJ-1	Higher values[b]	Lower values[b]	(Devic et al., 2011)
	0.84 μg ml^{-1}	0.42 μg ml^{-1}	(Masters et al., 2015)
	4.11 ± 5.88 ng ml^{-1}	3.86 ± 5.44 ng ml^{-1c}	(Kang et al., 2014)
AchE activity	0.36 ± 0.22 a.u.	0.19 ± 0.15 a.u.	(Fedorova et al., 2015)
micro-RNA (miR-874 and miR-145–3p)	No statistically significant difference	No statistically significant difference[c]	(Chen et al., 2020)
micro-RNA (miR-153 and miR-223)	Lower values[a]	Higher values[a,c]	(Cressatti et al., 2020)

PSP: progressive supranuclear palsy; α -syn$_{olig}$: alpha-synuclein oligomeric form; α-syn$_{tot}$: total alpha-synuclein; DJ-1: protein deglycase; a.u.: absorbance units.
[a]Statistically significant.
[b]Not statistically significant.
[c]HC subjects.
[d]PSP subjects.

which affects approximately 6 million people worldwide (Dorsey et al., 2018). It is characterized by dopaminergic neuronal loss in the pars compacta of the substantia nigra (SNpc) and by the presence of Lewy bodies (Fig. 3). The latter are due to the aggregation of monomeric alpha-synuclein (α -syn$_{mon}$) in its oligomeric form (α -syn$_{olig}$), followed by the conversion of these aggregates into mature amyloid fibrils (Kalia and Lang, 2015; Vekrellis et al., 2011). PD includes both motor (i.e., tremor, bradykinesia, rigidity and postural instability) and non-motor symptoms (such as psychosis, anxiety, dementia and depression), which could precede motor impairment by several years (Kalia and Lang, 2015; Schneider et al., 2017). Nowadays, the diagnosis of PD involves the use of the Unified Parkinson's Disease Rating Scale (UPDRS), which evaluates the mental and psychological state, as well as imaging techniques such as SPECT and PET or, in the case of the familial form, genetic sequencing (Gofton and Jog, 2008). An early diagnosis can be fundamental to limit the harmful effects of the disease. In this context, salivary biomarkers could represent a valid alternative to the current diagnostic tools. Among these, alpha-synuclein and DJ-1 are the most analyzed in scientific literature (Table 1).

Alpha-synuclein is a small protein localized in the presynaptic terminals of neuronal cells. In CSF, its concentration has already been widely studied and is known to be decreased in PD patients (Parnetti et al., 2011; Tokuda et al., 2006). Its salivary form resembles the results obtained with CSF, being decreased in patients with PD compared to cognitive healthy subjects (Al-Nimer et al., 2014; Devic et al., 2011; Shaheen et al., 2020; Vivacqua et al., 2016, 2019). It could also allow the differential diagnosis with progressive supranuclear palsy (PSP) (Vivacqua et al., 2019). Moreover, several studies focused on alpha-synuclein's oligomeric form (α -syn$_{olig}$) and the α -syn$_{olig}$/ α-syn$_{tot}$ ratio: both these parameters were significantly higher in patients with PD compared to healthy controls (Cao et al., 2019; Shaheen et al., 2020; Vivacqua et al., 2016, 2019). Vivacqua et al. speculated that these findings could be due to an alpha-synuclein altered turnover, which leads to accumulation of its oligomeric form (Vivacqua et al., 2016). Alpha-synuclein correlation with progression of the disease and cognitive problems has also been evaluated but there are conflicting results (Devic et al., 2011; Shaheen et al., 2020; Vivacqua et al., 2016).

DJ-1 is a highly studied protein correlated with the pathogenesis of PD and currently evaluated as a potential biomarker. Salivary levels of DJ-1 seem to be higher in PD than controls but these results were not statistically significant (Devic et al., 2011) or they may have been affected by the increase in total salivary proteins (Masters et al., 2015). However, different values could differentiate among disease stages and subtypes (such as the tremor dominant type, the akinetic-rigid dominant type and the mixed ones), as reported by Kang et al. (2014). Therefore, DJ-1 is probably not specific enough for the diagnosis of Parkinson's but it could be used to study the progression of the disease (Kang et al., 2014). Also AchE activity, which appeared to be increased and correlated to the progression of the disease (Fedorova et al., 2015), as well as micro-RNA expression (Cressatti et al., 2020 ; Chen et al., 2020) could be useful to diagnose PD (Table 2).

4. Amyotrophic lateral sclerosis

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disorder that affects motor neuronal cells (Fig. 4) and could have a limb onset, a bulbar onset or it may include only the upper or the lower motor neuron. This disease can lead to several symptoms, such as spasticity, fasciculation, fatigue, muscle atrophy, spastic dysarthria and dysphagia (Kiernan et al., 2011). Diagnosis of ALS is primarily clinical,
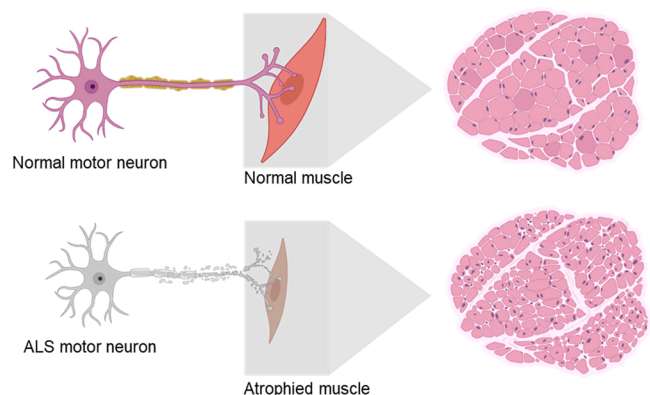
Fig. 4. Degeneration of motor neurons in amyotrophic lateral sclerosis leads to progressive skeletal muscle weakness and wasting.

Table 3

Salivary levels of biomarkers in Amyotrophic Lateral Sclerosis, Huntington's disease, and Multiple Sclerosis.

Biomarker	Patients	Healthy subjects	Reference
Amyotrophic Lateral Sclerosis			
CgA	12.58 ± 2.79 pmol ml^{-1}[d]	4.04 ± 2.04[f]	(Obayashi et al., 2008)
	6.36 ± 1.62 pmol ml^{-1}[e]	3.77 ± 1.90[c]	
Huntington's disease			
Htt protein	Cohort 1:	Cohort 1:	(Corey-Bloom et al., 2018)
	0.547 ± 0.079 ng ml^{-1} [g,a]	0.340 ± 0.046[c]	
	0.464 ± 0.133 ng ml^{-1} [h,b]		
	Cohort 2:	Cohort 2:	
	0.593 ± 0.078 ng ml^{-1} [g,a]	0.378 ± 0.068 ng ml^{-1}[c]	
	0.462 ± 0.055 ng ml^{-1} [h,b]		
BDNF	Lower values[a,g]	Higher values[a,c]	(Gutierrez et al., 2019)
	Lower values[a,h]		
uric acid	Lower values[a] ([g,k] and[g,h,l])	Higher values[a,c]	(Corey-Bloom et al., 2020b)
IL-6	Higher values compared to PM	Lower values[b,c]	(Corey-Bloom et al., 2020a)
	Lower values[a,h]		
Multiple Sclerosis			
sHLA II	386 ± 52 unit ml^{-1}	222 ± 18.4 unit ml^{-1}[c]	(Adamashvili et al., 2005)
	354 ± 42 unit ml^{-1}	222 ± 18 unit ml^{-1}[c]	(Minagar et al., 2007)
	821 ± 86 unit ml^{-1} (after 3 months[m])		
	776 ± 63 unit ml^{-1} (after 6 months[m])		
sHLA I	Mostly undetectable (11 of 13)	41 ± 2.8 ng ml^{-1} (41 of 53[c])	(Adamashvili et al., 2005)
FLC	31.6 (CI 17.4–45.8)[i]	5.2 (CI 3.7–6.6)[c]	(Kaplan et al., 2018)
	11.3 (CI 8.2–14.5)[j]	1.94 (CI 0.36–4.0) ($\kappa D + \lambda D$[c])	(Lotan et al., 2020)
	5.34 (CI 1.1–17.1) ($\kappa D + \lambda D$[i])	1.17 (CI 0.1–6.9) ($\kappa M + \lambda M$[c])	
	10.28 (CI 0.5–34.5) ($\kappa M + \lambda M$[i])		
	1.8 (CI 0.1–6.8) ($\kappa D + \lambda D$[j])		
	2.0 (CI 0.0–13.1) ($\kappa M + \lambda M$[j])		
TBARS	Higher by 51%	Lower[c]	(Karlík et al., 2015)
AGEs	Higher by 49%	Lower[c]	(Karlík et al., 2015)
AOPP	No statistically significant difference	No statistically significant difference[c]	(Karlík et al., 2015)
FRAS	Lower by 38%[a]	Higher values[a]	(Karlík et al., 2015)
TAC	Lower values[b]	Higher values[b]	(Karlík et al., 2015)

MS: Multiple Sclerosis; RRMS: relapsing/remitting form of multiple sclerosis; ALS: Amyotrophic Lateral Sclerosis; HD: Huntington's disease; sHLA I: soluble human leukocyte antigen class-I; sHLA II: soluble human leukocyte antigen class-II; FLC: immunoglobulin free light chains; TBARS: thiobarbituric acid reacting substances; AGEs: advanced glycation end-products; AOPP: advanced oxidation protein products; FRAS: ferric reducing ability of saliva; TAC: total antioxidant capacity; CgA: salivary chromogranin A; Htt protein: Huntingtin protein BDNF: brain-derived neurotrophic factor; IL-6: interleukin 6; IFN β-1a: interferon β-1a; κD: immunoglobulin kappa (κ) chain dimer; κM: immunoglobulin kappa (κ) chain monomer; λD: immunoglobulin lambda (λ) chain dimer; λM: immunoglobulin lambda (λ) chain monomer; CI: confidence interval.

[a] Statistically significant.
[b] Not statistically significant.
[c] HC subjects.
[d] Patients in a terminal stage.
[e] Moderate patients.
[f] Patients with tube-fed vascular dementia.
[g] Manifest patients.
[h] Pre-manifest patients (PM).
[i] Active MS patients.
[j] MS patients in remission.
[k] Male patients.
[l] Female patients.
[m] IFN β-1a.

and it is often complicated because ALS can resemble other neurological diseases (Hardiman et al., 2017).

The affective state of ALS patients can be related to Chromogranin A (CgA) values, as assessed by Obayashi et al. (2008) (Table 3) in ALS patients in a terminal stage of the disease, compared to moderate patients, patients with tube-fed vascular dementia and healthy controls. Thus, CgA may reflect the severity of ALS and it may be useful to develop psychophysiological targeted treatments, therefore improving patients' quality of life (Obayashi et al., 2008).

5. Huntington's disease

Huntington's disease (HD) is a genetic inherited disorder caused by expansions of the huntingtin gene (Htt), which encodes for the Huntingtin protein (Ha and Fung, 2012). More specifically, it is an autosomal dominant disease caused by at least 36 repetitions of CAG trinucleotide on the short arm of chromosome 4 (Nance, 2017) (Fig. 5). HD includes several clinical manifestations, such as motor, neuropsychiatric and cognitive symptoms. Among these, the chorea is a typical symptom characterized by abnormal involuntary and unpredictable movements of the face, limbs and trunk (Snowden, 2017). Nowadays, the diagnosis of this disease consists of anamnesis, clinical evaluation and genetic tests (Ha and Fung, 2012; Snowden, 2017).

The assessment of biomarkers, such as Htt protein, in CSF and blood samples is not widespread because of the invasiveness of these methods and low Htt levels. On the contrary, higher Htt protein values were detected in saliva, as stated by Bloom et al., who used ELISA test to compare healthy controls and HD patients. Total Htt protein levels were
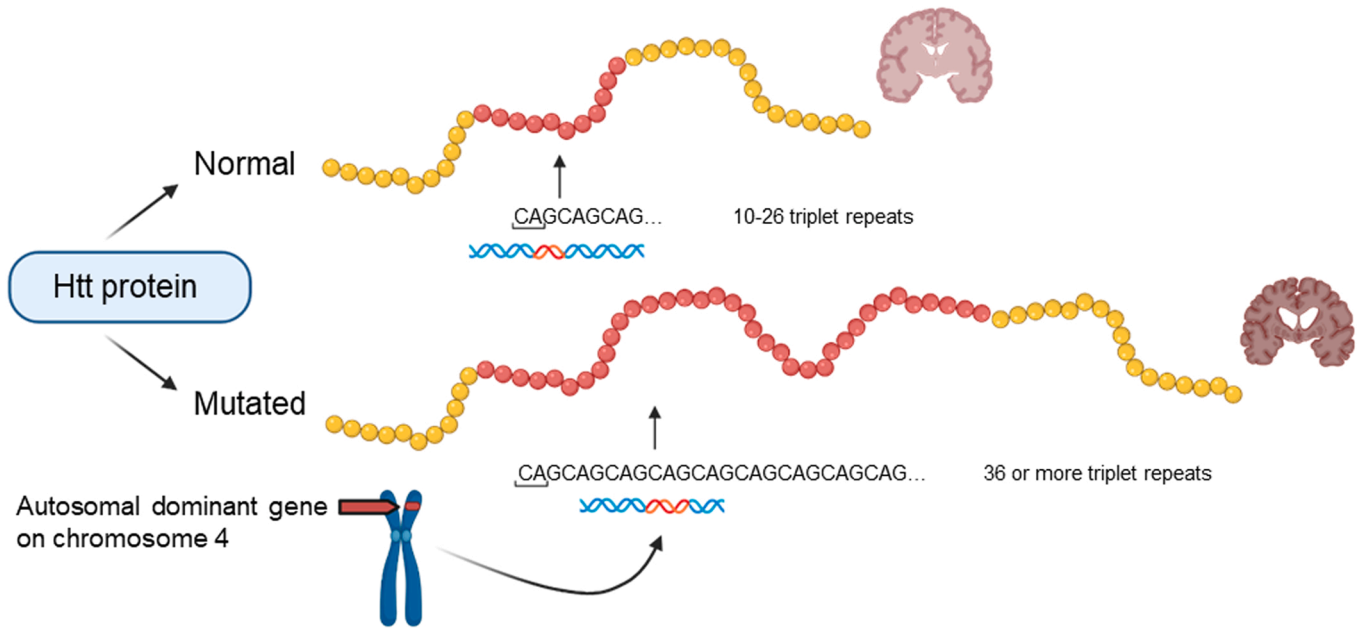
Fig. 5. Pathogenesis of Huntington's disease and ventricular enlargement in a coronal cut of a pathological brain.
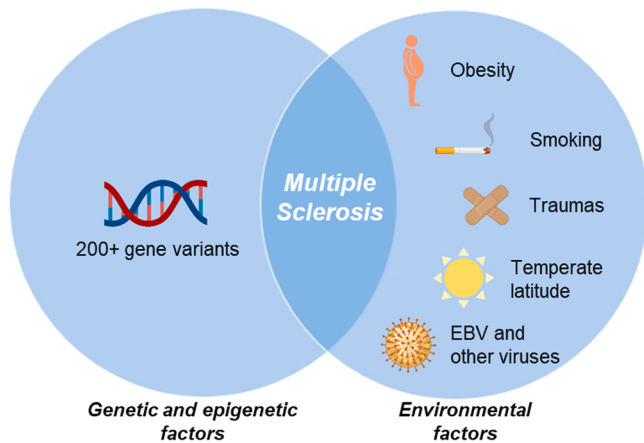

Fig. 6. Risk factors of multiple sclerosis.

significantly increased in patients compared to healthy controls, while the pre-manifest group showed higher values than controls but the difference was not statistically significant (Corey-Bloom et al., 2018) (Table 3).

Furthermore, other biomarkers are currently being studied for HD diagnosis. Among these, brain-derived neurotrophic factor (BDNF) showed to be lower in both pre-manifest and manifest HD patients compared to controls, thus representing a potential biomarker for early diagnosis of Huntington's disease (Gutierrez et al., 2019) (Table 3). Also, uric acid (UA) and interleukin-6 (IL-6), although probably not specific, have been studied as potential biomarkers for HD diagnosis (Table 3). Particularly, as concerns uric acid, different results emerged between males and females. The first ones showed decreased values of UA in salivary samples of manifest patients compared to controls, while lower levels of UA were found in both salivary and blood samples of women with pre-manifest or manifest HD compared to healthy controls. These results could highlight the role of gender in HD pathophysiology (Corey-Bloom et al., 2020b). Regarding IL-6, it showed increased levels in HD patients compared to both pre-manifest patients and healthy controls. Moreover, it resulted correlated with clinical manifestations of the disease, highlighting the link between inflammatory biomarkers

detected in saliva and the neurodegenerative process of central nervous system (Corey-Bloom et al., 2020a).

Considering that CAG repeat expansions can be detected with genetic testing and be identified long before symptomatic presentation in families carrying the mutation, biomarkers may be relevant for de novo cases only, thus, given the rarity of the condition, of limited usefulness in clinical practice.

6. Multiple sclerosis

Multiple sclerosis (MS) is a demyelinating disease involving central nervous system and mainly affecting women and subjects between 20 and 40 years old. It is an inflammatory, autoimmune disorder caused by a complex interaction between environmental and genetic factors (Oh et al., 2018; Katz Sand, 2015), that are summarized in Fig. 6. MS is characterized, in the majority of cases, by acute phases followed by remission of symptoms. Neurological deficits include motor and sensory systems, but other symptoms may occur, including cognitive and psychiatric symptoms, fatigue, muscle spasm, vision problems, deficit in attention and depression (Chiaravalloti and DeLuca, 2008; Chwastiak et al., 2002; Rae-Grant et al., 1999; Rizzo et al., 2004). Clinical examination, magnetic resonance imaging (MRI) and CSF analysis for the detection of oligoclonal bands currently allow the diagnosis of MS (Oh et al., 2018). Nevertheless, the use of salivary biomarkers could be useful to make a differential diagnosis with other demyelinating syndromes (Miller et al., 2008) and to predict the course of the disease and the response to treatment, thus allowing the choice of the most appropriate pharmacological approach.

Particularly, the soluble form of the Human Leukocyte Antigen (sHLA) has been studied in MS patients and controls (Table 3). It emerged that sHLA class II is increased in patients with Relapsing Remitting Multiple Sclerosis (RR-MS) (Adamashvili et al., 2005; Minagar et al., 2007), reaching values similar to the CSF ones (Adamashvili et al., 2005). Moreover, it seems to be higher also after interferon β1a treatment and this could indicate a good response to the drug (Minagar et al., 2007). On the contrary, sHLA-I had very low levels in saliva, sometimes even being undetectable (Adamashvili et al., 2005; Minagar et al., 2007). Moreover, immunoglobulin free light chains (FLC) levels, detected by semi-quantitative western blot analysis, could discriminate between patients in the active phase of the disease and patients in
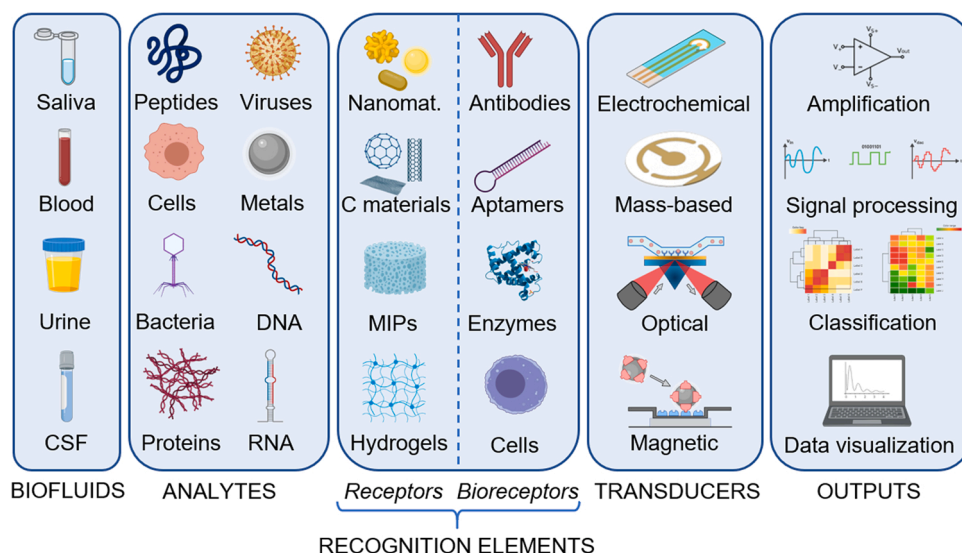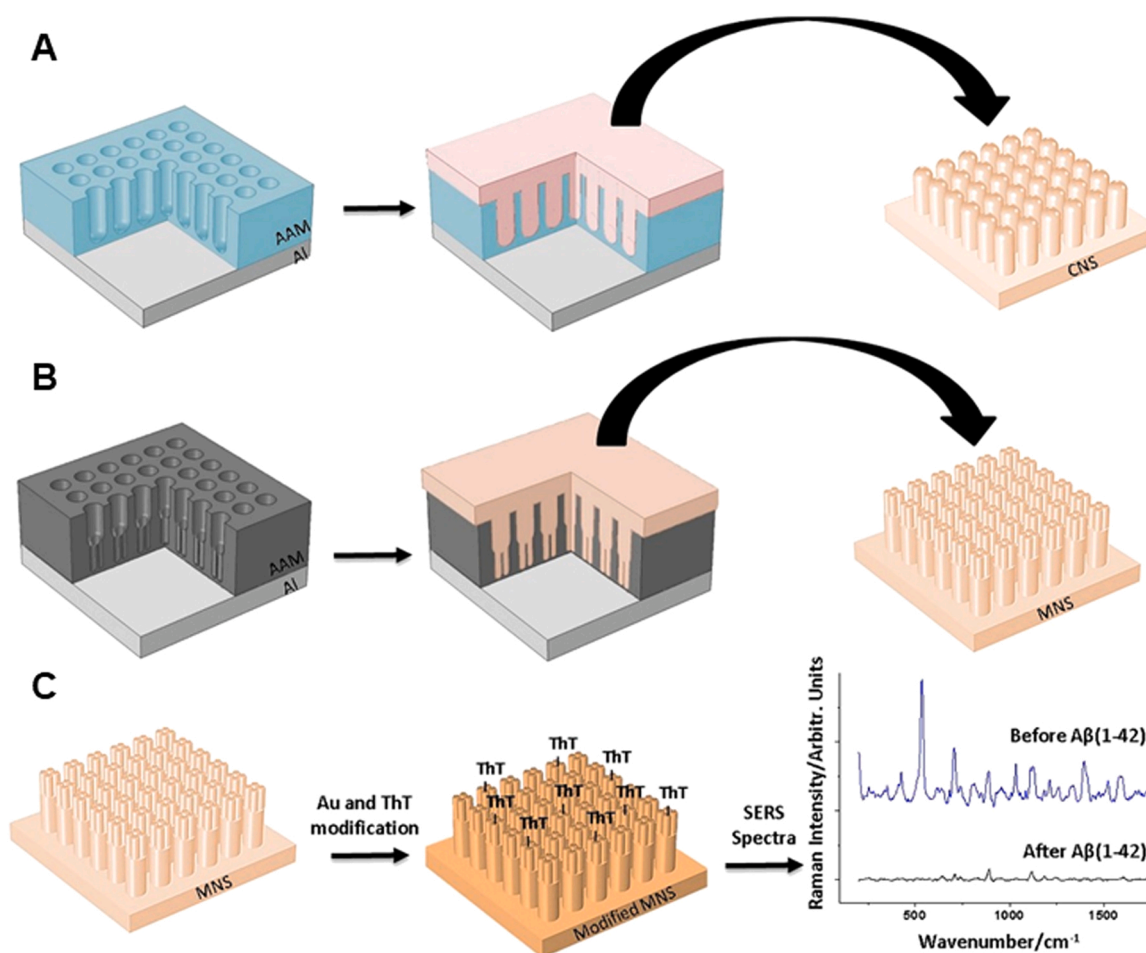
Fig. 7. Elements of a biosensor.

Fig. 8. The schematic illustration of (A) cylindrical nanopillar surfaces (CNS) and (B) multibranched nanopillared surfaces (MNS) film formation from anodized alumina molds (AAMs) having cylindrical and multibranched columnar structures, respectively. (C) Au-coated MNS films were used to chemisorb thioflavin-T (ThT), the SERS signal of which attenuates when treated with $A\beta_{1-42}$ in a concentration dependent manner.
Reproduced with permission from Altuntas and Buyukserin (2018).

remission. The first group showed higher levels of FLC in saliva, both compared to controls and to patients in remission of MS (Kaplan et al., 2018; Lotan et al., 2020) (Table 3).

Potential markers studied in patients as compared with controls are summarized in Table 3. Karlik et al. analyzed saliva and blood of MS patients and healthy controls and they quantified markers of protein oxidation, lipoperoxidation and carbonyl stress (Karlík et al., 2015). As concerns saliva, the study showed that thiobarbituric acid reacting substances (TBARS) and advanced glycation end products (AGEs) were higher in the salivary samples of MS patients than those of controls, while advanced oxidation protein products (AOPP), which were higher in the blood of MS patients, were unchanged in saliva. Moreover, the levels of salivary oxidative stress biomarkers can be influenced by oral pathologies and circadian rhythm, so these parameters should be considered (Karlík et al., 2015). The authors also analyzed Ferric Ion Reducing Ability of saliva and plasma (FRAS/P) and Total Antioxidant Capacity (TAC). Both salivary values were lower in MS patients, but only the first one showed a significant difference compared to healthy controls (Karlík et al., 2015).

It has however to be acknowledged that current criteria for MS are accurate enough to distinguish between cases and controls, even in early phases of the disease. Instead, biomarkers for differential diagnosis among demyelinating disorders such as neuromyelitis optica or primary leukodystrophy disorders, would be more useful in a clinical setting. Therefore, future search for saliva biomarkers should be focused on the whole spectrum of demyelinating disorders.

7. Biosensors and wearable bioelectronic platforms for the detection of NDDs biomarkers

The majority of NDDs and demyelinating diseases are nowadays diagnosed through conventional methods such as genetic testing, cognitive assessments, imaging, or CSF analysis (DeKosky and Marek, 2003; Kuhlmann et al., 2008; Stoessl, 2012; Tillema and Pirko, 2013). These methods, even though reliable and widely accepted, show several drawbacks including complex protocols, high costs for equipment and trained personnel, as well as invasive biofluid collection procedures (i. e., CSF sampling) (Ausó et al., 2020; Serafín et al., 2020; Wei et al., 2018).

Biosensors are a subclass of chemical sensors, which convert biochemical data into an analytical signal that is used to monitor physiological and chemical analytes. The schematic diagram in Fig. 7 shows the various elements of a biosensor. The biosensor can target analytes of different nature and size, through a specific biochemical recognition element, the bioreceptor. A physicochemical transducer translates the biochemical event (i.e., binding, protein folding, etc.) into a meaningful measurable signal. The readout circuitry, if present, process the obtained signals and transmit them to a secondary device to perform data analysis and processing (Kim et al., 2019; Mani et al., 2021; Yang and Gao, 2019; Zheng et al., 2021).

Biosensors are deemed to offer several advantages over traditional screening procedures, such as a rapid time-to-response, ease of use, low-cost and low-power requirements, portability, and proximity to the patient through the exploitation of point-of-care (POC) devices (Goldoni et al., 2021; Kim et al., 2019; Mani et al., 2021; Yang and Gao, 2019; Zheng et al., 2021). To date, researchers are able to develop extremely sensitive and selective biosensors that can be used to screen biomarkers in low concentration and complex biomatrices (Bandodkar and Wang, 2014; Falk et al., 2020; Sharma et al., 2021), such as blood (Chen et al., 2017; Lei et al., 2017; Liu et al., 2019; Makhaeva et al., 2007; Ouyang et al., 2021), urine (Akyilmaz et al., 2003; Della Ventura et al., 2019; Liao et al., 2006), tears (Ballard et al., 2020; Chu et al., 2011; Elsherif et al., 2018; Kownacka et al., 2018; Shum et al., 2009; Thomas et al., 2012), sweat (Bandodkar et al., 2013; Jia et al., 2013; Kim et al., 2011, 2016; Mishra et al., 2018) and saliva (Arakawa et al., 2016; Kim et al., 2015, 2014b; Tseng et al., 2018; Viswanath et al., 2017). Despite this, an

accurate and reliable detection of neurodegenerative biomarkers in body fluids is still at an early stage (Gottsauner et al., 2020; Shui et al., 2018).

Being the gold standard of conventional clinical examinations, some research groups have worked on the development of biosensors for the detection of neurodegenerative biomarkers in CSF (Gottsauner et al., 2020; Haes et al., 2005; Shui et al., 2018; Song et al., 2018), despite the invasiveness of the sampling procedure. A much larger share of the research articles on biosensors for NDDs, however, have been validated clinically on human serum or plasma. In certain cases, instead, the developed biosensors have been tested in an artificial medium, before moving on to the clinical application with a real biofluid. In some other cases, biosensors have been developed to detect biomarkers of NDDs in more unconventional fluids, such as sweat, urine and saliva (Altuntas and Buyukserin, 2018; Lau et al., 2015; Shui et al., 2018; Sonuç Karaboğa and Sezgintürk, 2021). These unconventional fluids in some cases, however, result being more promising than serum for diagnostics. As a recent demonstration of this, Adam et al. (2021) reported the development of an electrochemical sensor based on gold nanorods (Au NRs) for the detection of alpha synuclein, a PD biomarker. They concluded that alpha synuclein should be analyzed in other fluids, including saliva, since serum does not have a reasonable amount of the biomarker for an accurate determination of the disease.

Altuntas and Buyukserin (2018) reported the development of a biosensor to detect beta-amyloid peptide (Aβ_{1-42}), a biomarker associated with the onset of Alzheimer's disease. The platform (Fig. 8) is based on 20–nm Au coated multibranched nanopillared (MNS) polymeric films modified with Thioflavin-T (ThT). The ThT-modified MNS films demonstrated a gradual ThT SERS signal suppression when incubated with increasing concentrations of Aβ_{1-42} in aqueous solution, reaching a detection limit of 0.5 pg ml^{-1} with a linear dynamic range between 0.5 pg ml^{-1} and 100 ng/ml. The sensing performances were also investigated using peptide solutions prepared with artificial saliva, showing a reduction of 75% of the Raman intensity after immersion in 10 pg ml^{-1} solutions of Aβ_{1-42} in saliva, compared to the protein-free saliva counterpart. On the other hand, the immersion in 10 pg ml^{-1} of myoglobin in saliva caused a reduction of only 15% of the Raman intensity, confirming the specific interaction of ThT with the amyloid forming peptide.

Sonuç Karaboğa and Sezgintürk (2021) reported the detection of DJ-1 protein, developing an electrochemical-based single-use neuro-biosensor that is reported in Fig. 9. The nanocomposite biosensor is based on 11-amino-1-undecanethiol (11-AUT)-modified polyethylene terephthalate coated indium tin oxide (ITO-PET) electrodes that have been doped with anti-DJ-1 antibody functionalized multiwalled carbon nanotube (MWCNT)-gold nanoparticles (AuNPs). The biosensor was able to reveal DJ-1 in the range 4.7–4700 fg/ml with a limit of detection of 0.5 fg/ml in solution, exhibiting high selectivity versus presence of other proteins, as Tau-441 (a biomarker for AD), alpha-synuclein (another biomarker for PD), and HSP-70. Eventually, the sensing performances were also evaluated using real saliva samples as complex biomatrices, demonstrating the possibility to detect DJ-1 concentration ranging from ~240 to ~5100 fg/ml.

The design of smart systems based on biosensors promises the realization of portable, flexible, multifunctional, and efficient operation apparatus, which enable realization of real-time, fast, and in vivo detection of biomarkers of interest also in saliva (Kim et al., 2019; Malon et al., 2014; Min et al., 2021; Mishra et al., 2016; Senf et al., 2020; Zheng et al., 2021). In fact, compared with blood acquisition in traditional analysis, extraction of saliva is non-invasive and friendly to patients, avoiding cross infection and privacy problem (such as urine collection). Fig. 10 summarizes the most recent efforts in the realization of wearable biosensing platforms for salivary analysis enabling non-invasive diagnostics.

The use of mouthguard-based devices for saliva testing is a new technology proposed in the recent years (Arakawa et al., 2016, 2020;
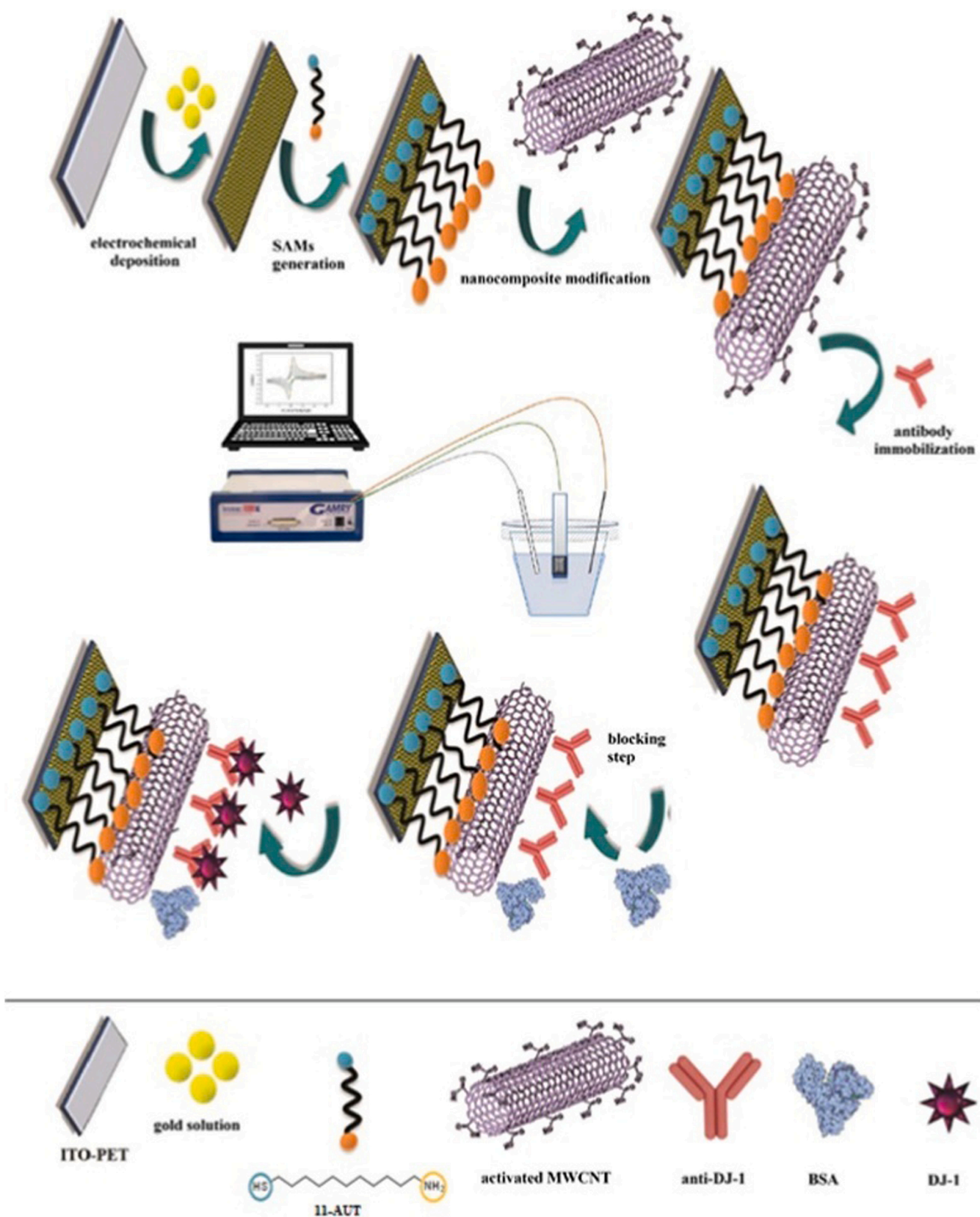
Fig. 9. Representation of the various steps in the development of the neurobiosensor, and description of the building elements. Reproduced with permission from Karaboga and Sezginturk (2021).
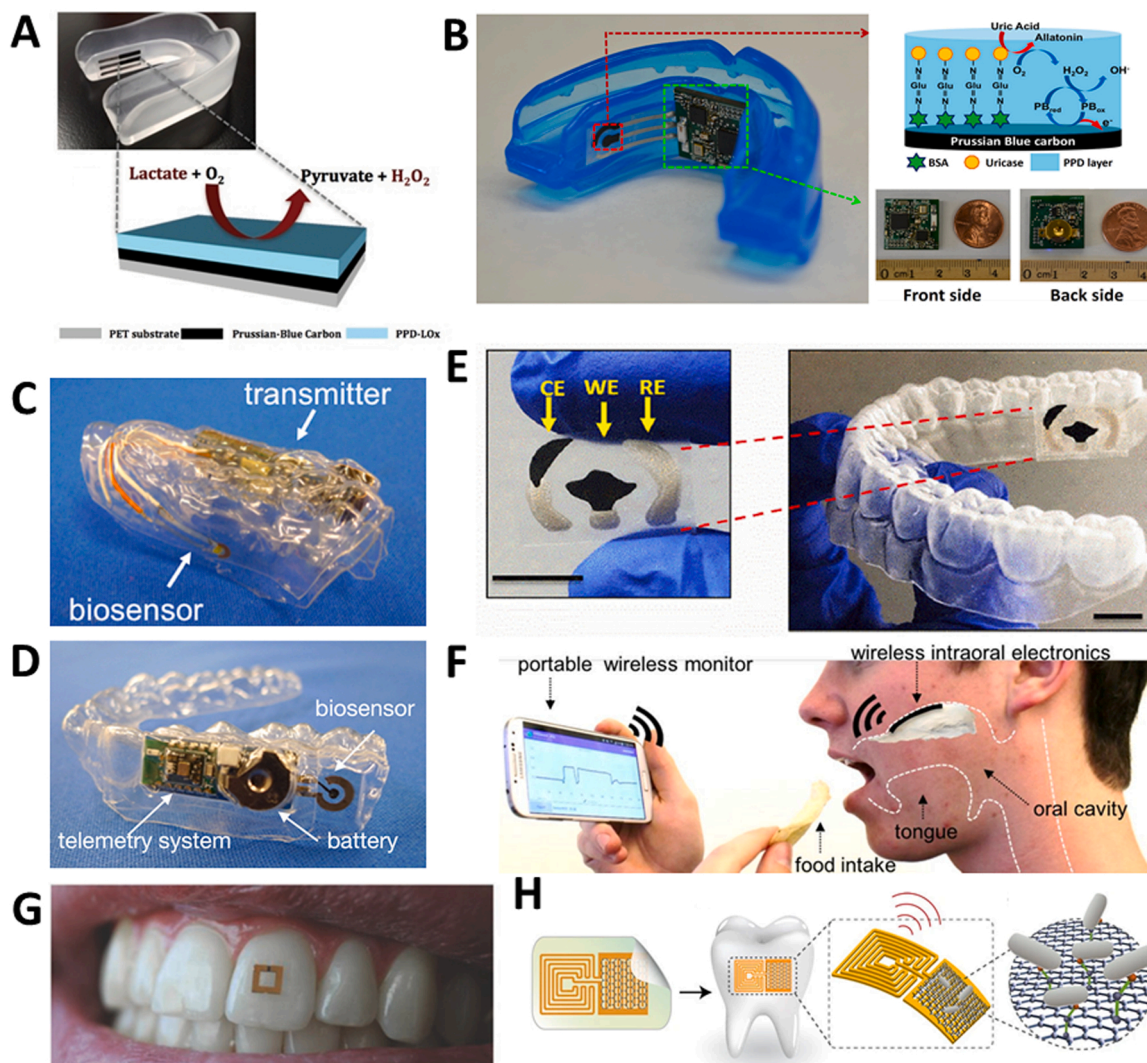
Fig. 10. Summary of wearable intraoral biosensors for salivary analysis. (A) Lactate biosensor on a mouthguard. (B) Mouthguard with biosensor and integrated electronics for real-time uric acid detection. (C) and (D) Glucose biosensing telemetry system. (E) Mouthguard with screen printed electrodes for N-Carboxymethyl-lysine detection. (F) Hybrid flexible bioelectronic platform for sodium monitoring. (G) Tooth mounted RF hydrogel biosensor. (H) Graphene-based biosensor for pathogen's detection.
(a) Reproduced with permission from Kim et al. (2014a) (b) Reproduced with permission from Kim et al. (2015). (c) Reproduced with permission from Arakawa et al. (2016). (d) Reproduced with permission from Arakawa et al. (2020). (e) Reproduced with permission from Ciui et al. (2019). (f) Reproduced with permission from Lee et al. (2018). (g) Reproduced with permission from Tseng et al. (2018). (h) Reproduced with permission from Mannoor et al. (2012)

Ciui et al., 2019; Kim et al., 2015, 2014b). For example, Kim et al. (2014b) reported the detection of salivary lactate, a specific biomarker for respiratory insufficiency, heart failure, and genetic diseases, using a wearable biosensor via the integration of a printable enzymatic electrode on a mouthguard (Fig. 10a). Later, the same research group (Gottsauner et al., 2020; Kim et al., 2015) continued to develop an anatomically miniaturized instrumented mouthguard platform for salivary uric acid monitoring, using a flexible electrode screen-printed on PET substrate, equipped with a Bluetooth Low Energy transceiver, potentiostat and microcontroller (Fig. 10b). Arakawa et al. (2016) instead realized a wearable mouthguard biosensor based on a telemetry system for the detection of salivary glucose (Fig. 10c). The biosensor is based on a two-electrode (Pt and Ag/AgCl) system featuring an enzymatic membrane deposited on the Pt electrode. Later the same group (Arakawa et al., 2020) improved the original design by including a selective membrane on top of the enzymatic layer to prevent a rapid biofouling of the sensing surface. They also demonstrated real-time monitoring of glucose concentration when the novel biosensor (Fig. 10d) is directly inserted into a patient mouth for the clinical

validation of the device. Similarly, Ciui et al. (2019) integrated a screen printed three-electrode system on a flexible foil onto a mouthguard (Fig. 10e), to determine N-Carboxymethyl-lysine (CML) in saliva, a typically advanced glycation end product, related to oxidative stress and long-term protein damage in diabetes, ageing and atherosclerotic plaques. Although not being technically considered a mouthguard, the intraoral biosensing device proposed by Lee et al. (2018) still fits the category of removable wearable devices for non-invasive biosensing (Fig. 10f). The bioelectronic platform consists of a highly integrated hybrid flexible architecture based on the seamless integration of hard and soft components, allowing non-invasive real-time sodium detection to facilitate the management of hypertension for at-risk patients.

Steering from the use of mouthguard-based devices, Tseng et al. (2018) recently proposed a wearable biocompatible biosensor based on a conformal radiofrequency (RF) construct composed of an active layer, namely porous silk film, and a modified PNIPAM hydrogel (Fig. 10g). The hydrogel is encapsulated between two reverse-facing split ring resonators mounted on a tooth and capable of wireless monitoring of foods during ingestion, measuring fluid properties such as alcohol

content, salinity, sugars, pH and temperature in vivo. Lastly, Mannoor et al. (2012) was the first to propose a biosensing platform directly attached on the tooth enamel for the detection of a specific bacterial target. The nanosensor is based on a graphene sensing layer printed onto a water-soluble silk thin-film substrate and then integrated with electrodes patterned with an inductive coil antenna (Fig. 10h). This passive patch-like biosensor had the advantages of being free from discrete and rigid components and it could easily be interrogated with the supplement of an external reader to be put in proximity of the nanobiosensor.

Molecular diagnostics for NDDs can greatly benefit from the flourishing field of biosensing, especially in the case of multiplexed biosensors, that can give more accurate diagnostic outcomes through the detection of a set of relevant biomarkers instead of a single one. The detection of a single marker is, indeed, sufficient to make a precise diagnosis only in rare occasions, examples being the monitoring of glucose for diabetic patients and the monitoring of sodium for people suffering from hypertension. The onset and progression of neurodegenerative diseases cannot be traced back to the signature of a single marker but it is instead signaled by the overexpression of a panel of markers. In this regard, the development of biosensing platforms that can detect simultaneously a set of relevant markers is highly sought. As of now, however, only a few examples of multiplexed biosensing platforms can be found in literature for applications in neuroinflammation and neurodegeneration (Chen et al., 2015; Xia et al., 2010; Liu et al., 2014). Moreover, none of these studies have saliva as the biofluid of choice for the biosensing application, thus reinforcing the idea that further development is needed in the field of salivary diagnostics paired with advanced biosensing. The development of advanced diagnostic tools paralleling the discovery of increasingly more predictive set of biomarkers is therefore expected to have a significant social impact in early diagnosis and treatment of NDDs and demyelinating diseases.

8. Conclusions and future perspectives

Saliva analysis showed to be a promising tool to detect biomarkers related to NDDs and demyelinating diseases that are associated with an aging population. The use of these platforms may allow an early, non-invasive, and simpler diagnosis, potentially improving the life of many patients and reducing the economic and social burden of these diseases (Abati et al., 2020; Chakraborty et al., 2019; Keshavarzi et al., 2017). Its use in clinical practice today is limited due to the absence of standardized methods of collection and analysis of salivary samples, as well as the lack of specific ranges of clinical relevance that can differentiate patients from controls based on the analysis of a single biomarker.

Regarding AD, we acknowledge that the majority of studies on salivary biomarkers have been performed on patients with overt dementia, who can be diagnosed clinically without the aid of biomarkers. Therefore, future studies on cohorts of prodromal (or even preclinical) AD are needed to confirm the possible use of such measurements as early biomarkers. Similarly, in ALS there are few studies, carried out in end-stage patients compared to moderate, but not in early phases of the disease, raising doubts on their usefulness for early detection. However, more broadly speaking, salivary biomarkers have many potential applications: they could still be widely employed in research settings, such as clinical laboratories, where they could be useful in investigating the effects of specific treatments. The peculiar traits of salivary diagnostics enabled by biosensors being its non-invasive nature and ease of repetition on a large scale makes it the perfect candidate for large-scale clinical trials. Moreover, the low cost of the manufacturing processes associated with the development of most biosensors make them attractive for low resource settings.

Literature preliminary results and the development of advanced diagnostic tools, such as biosensors, represent an optimal starting point for future research aimed to find a valid alternative to current diagnostic methods. Moreover, the rise of wearable biosensors and portable POC devices could help increase the patients' compliance toward the

frequent use of diagnostic tools, offering additional advantages compared to standard laboratory analysis, such as the evaluation of the circadian rhythm of specific biomarkers. Overall, the opportunity to collaborate with clinicians by sharing continuous medical data is expected to enable better predictions and more accurate diagnosis in the near future, and we expect biosensors to play a pivotal role in this technological transition. Therefore, the scope of this review is to stimulate further research in this direction, both on the clinical side toward the discovery and validation of more accurate biomarkers, and on the technological side, through the development of non-invasive ultrasensitive biosensing platforms for the diagnosis of age-related neurodegenerative diseases.

CRediT authorship contribution statement

R.G.: Conceptualization, Visualization, Writing-Original Draft, Writing-Reviewing and Editing, **C.D.**: Visualization, Writing-Original Draft, **E.B.**: Visualization, Writing-Original Draft, **F.I.**: Visualization, **A.P.**: Writing-Original Draft, Writing-Reviewing and Editing, **L.S.**: Writing-Reviewing and Editing, **D.G.**: Conceptualization, Writing-Reviewing and Editing, Supervision, **G.T.**: Conceptualization, Supervision, Project Administration.

Declaration of Competing Interest

Acknowledgments

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