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Review

Proteomics as a new paradigm to tackle Parkinson's disease research challenges

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ABSTRACT

Disease-modifying therapies capable to stop or slow Parkinson's disease (PD) progression are still elusive due to severe shortcomings in the understanding of PD etiopathogenesis as well as limitations in routine clinically-based diagnosis precluding PD detection during its early course. Proteomics has recently emerged as one of the most attractive approaches to unravel the complex nature of PD processes and investigate PD potential biomarkers. In contrast to traditional candidate-based studies, it offers global and high-throughput strategies to systematically analyze proteins – the pathological effectors themselves – without the need to establish *a priori* hypotheses. This review aims to summarize the latest advances in PD research in the context of proteomics. After an overview of some methodological aspects, the most recent PD-related findings will be discussed together with the limitations and perspectives of current proteomic workflows.

Contents

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1. Introduction

Parkinson's disease (PD) is the most common neurodegenerative movement disorder, affecting adult individuals of all races and culture. The progressive deterioration of motor function, manifested clinically by various degrees of tremor at rest, rigidity, slowness of movement (bradykinesia) and postural instability, appears after a significant loss of dopaminergic neurons in the substantia nigra (SN) pars compacta has been reached. Nigral neurodegeneration together with the presence of distinctive intracytoplasmic inclusions referred to as Lewy bodies (LB) in the surviving neurons are the two invariant pathological hallmarks of PD which are mandatory to establish a definitive diagnosis at autopsy. Non-motor symptoms encompassing cognitive decline, anxiety, sleep disturbances, or autonomic impairment are increasingly recognized to be part of the PD clinical spectrum and may result from the vulnerability of selected neuronal populations in numerous regions of the central and autonomous nervous systems.

Altogether, PD results in major functional disabilities impacting quality of life, working capacity and life expectancy with mortality rates being nearly doubled in PD versus aged-matched subjects [1–3]. Despite the remarkable efficacy of dopamine replacement therapy to alleviate motor symptoms and improve patients' quality of life, PD remains incurable without any treatment available to modify or stop the disease's rampage through the nervous system. The estimated direct and indirect costs related to the illness ranks high among brain disorders, amounting up to 13.9 billion euros in Europe for the year 2010 alone [4]. The number of PD cases, which currently approximates 1.2 million in Europe (0.3% of the general population) and 1 million in the USA, is expected to double by year 2030 along with the increase of life expectancy in the Western populations [4–6]. In the absence of any disease-modifying therapy yet, the socioeconomic and financial burdens incurred by PD will continue to grow and defy our healthcare system over the coming decades.

Before any preventive or curative intervention could be designed, a clear and detailed understanding of the molecular mechanisms underlying neurodegeneration in sporadic PD is required. However, despite decades of research, this is definitely not the case yet. Many mechanisms have been shown to sensitize neurons to death, including impairment of protein degradation systems, mitochondrial dysfunction and oxidative stress, inflammation, excitotoxicity or enhanced apoptosis. In all likelihood, more than one of these, and possible many others, might be at work in PD but the precise combination and temporal succession of the molecular events leading to cell death remain to be disentangled.

Thus far, research into PD pathogenesis has heavily relied upon toxic and transgenic animal models, the engineering of which has derived from rare neurotoxin-induced and monogenic forms of parkinsonism in humans. However, these hypothesis-driven approaches have demonstrated major limitations, casting serious doubts about the validity of such models to address the complexity of PD pathogenesis. The recent emergence of more global, unbiased and hypothesis-free disciplines such as GWAS and "omics" may provide new research paradigms to explore PD pathogenesis and PD biomarkers, which may respectively pave the way for original neuroprotective or neuroregenerative therapeutic targets and offer early and accurate diagnostic tools. After reappraising some key aspects of PD neuropathology and etiopathogenesis, this review aims to summarize the ultimate advances in PD research in the context of proteomics. We will glance over proteomics techniques from sample preparation to mass spectrometry (MS) analysis before examining the most recent PD-related findings, limitations and future directions.

2. PD neuropathology: the disease substrates

2.1. Neuropathological hallmarks

Most available evidence suggests that the lesional core of PD pathology is the damage of dopaminergic cells in the SN pars compacta [7], which results in dopamine (DA) depletion in the striatum and destabilization of the basal ganglia (BG) motor control loops [8]. Nigral neurodegeneration is thus unambiguously linked to motor symptoms, which first become apparent when about 80% of striatal dopaminergic terminals and 50–60% of nigral dopaminergic cell bodies are already lost [9,10]. In the SN, neuronal loss is greater in the calbindin D_{28K}-poor compartments termed nigrosomes than in the calbindin D_{28K}-enriched matrix. The degree of neuronal loss is related to disease duration and follows a stereotyped spatiotemporal progression (from the more caudal nigrosome N1 > N2 > N4 > N3 to the more rostral nigrosome N5) [11] consistently observed across PD patients and differing from normal aging or other neurodegenerative disorders [7]. While neuronal loss is particularly severe within the SN ventrolateral tier, involvement of other midbrain dopaminergic cell populations (medial and medioventral, A8, substantia nigra pars lateralis, central gray substance) is less pronounced and may rather reflect some physiological aging-related decline [12].

Surviving nigral neurons frequently exhibit cytoplasmic protein inclusions referred to as LB or Lewy neurites if located in neuronal processes, which contain, among many others proteins, misfolded α-synuclein (α-SYN) and ubiquitin (Ub) [13]. It is still unclear if LBs themselves are the pathological entities interfering with normal cell function, if they represent a cytoprotective mechanism similarly to aggresomes or a failed attempt to eliminate cytotoxic proteins such as misfolded α-SYN. The percentage of LB-bearing nigral cells appears to be stable over time (3.6% in average), suggesting that they are eliminated as the disease progresses when the afflicted neurons die. Thus, in the SN at least, LB may be closely related to nigral neuronal loss [14]. Current knowledge on LB structure, formation, composition and role in cell death is still limited and reviewed elsewhere (in [15]). Of note, LBs are not specific for PD, as they are found in other forms of parkinsonism collectively termed "synucleopathies" (i.e., dementia with LB, multiple system atrophy), in Alzheimer's disease (AD), as well as incidentally in aged people [16].

2.2. Extranigral aspects of PD and Braak staging

Neuronal loss and LB formation are neither confined to the midbrain and the SN, nor restricted to the dopaminergic neurochemical system. Based on neuropathological studies, PD is now rather viewed as a multisystem disorder affecting numerous neuronal populations both in the central and peripheral nervous systems [17]. Dopaminergic neurons found outside the midbrain are unequally vulnerable to PD, partially lost in the retina [18] and enteric nervous system [19] while relatively spared in the

hypothalamus or bone marrow [20]. Noradrenergic (i.e., locus coeruleus), cholinergic (i.e., dorsal motor nucleus of the vagus nerve (DMV), nucleus basalis of Meynert), serotoninergic (i.e., raphe nuclei) or glutamatergic (i.e., amygdala, cortex) systems are also affected in anatomical regions of predilection within the brain as well as nerve and ganglia of the autonomic system [17]. Clinico-pathological correlations suggest that the neurodegenerative process extension beyond the BG structures may be responsible for numerous non- motor signs not attributable to nigrostriatal degeneration such as sleep disorders, dementia, depression and autonomic dysfunction (i.e., cardiovascular, gastrointestinal) [21]. Table 1 provides a non-exhaustive overview of susceptible CNS and ANS neuronal populations affected in PD, together with their known or putative clinical correlates.

PD pathology requires years to reach its full extent throughout the nervous system and the temporal relationships of the lesions are still not well established. Braak and co-workers proposed a neuroanatomical staging system based on α-SYN immunoreactivity distribution in the brains of PD patients and clinically asymptomatic incidental Lewy pathology cases. The authors predicted that PD pathology follows a stereotyped and selective caudo-rostral progression within vulnerable structures of the CNS (Table 1). In this scenario, the disease begins in the DMV and in the olfactory bulb (Braak 1), ascends in the brainstem to reach the raphe nuclei and the locus coeruleus (Braak 2) before affecting the SN (Braak 3). Finally, in later stages (Braak 4–6), the disease enters the temporal mesocortex and eventually the neocortex. Stage 1 and 2 are considered as pre-motor stages, with motor symptoms emerging only in stage 3 when SN neurodegeneration begins [17,22].

The predictive validity of Braak's concept of neuropathological staging has been somehow disputed as it does not seem to correlate with PD clinical severity and duration [23]. In fact, there is a considerable variability in the temporal sequence and topographical distribution of Lewy pathology among patients. Some studies have reported cases of aged individuals dying with Braak stages 4–6 without any clinical record of neurological impairment [24–26]. Moreover, the relationship between Lewy pathology and neuronal dysfunction or death is still uncertain, representing an additional challenge for the Braak's hypothesis. Although Braak's staging might require further clinical and pathological validation, it is still widely accepted as it broadly concurs with clinical observations and might be accurate in about 80% of the cases [27]. A more sensitive PD staging system might include neurodegeneration patterns in addition to Lewy pathology.

2.3. Is PD a prion-like disorder?

Braak and co-workers suggested that an unknown environmental insult initiates the pathological process, which may spread trans-synaptically from one susceptible brain region to another via

Table 1
Anatomical sites of PD pathology and clinical correlates (adapted from [28]).

Anatomical site	Neuro-transmitter	Lewy pathology	Neuronal loss	Braak stage	Clinical symptoms	Ref.
Autonomic nervous system						
Ganglia and nerve fibers						
■Gastroesophagal						
○Submandibular gland	ACh	✓	~		Sialorrhea, dysphagia	[29]
○Myenteric and submucosal plexus (i.e., oesophagus, stomach, colon)	ACh, DA	✓	~		Esophageal and gastric dysfunction, constipation	[30–32]
■Sympathetic chain						
○Cardiac plexus	NA	✓	✓		Cardiac denervation, orthostatic hypotension	[33–35]
■Adrenal gland	A, NA	✓	~		Autonomic dysfunction, hypotension?	[36]
Spinal cord						
■Intermediolateral n.	ACh	✓	✓		Orthostatic hypotension	[37]
Medulla						
■Dorsal motor n. of glossopharyngeus and vagus (IX/X)	ACh	✓	✓	1	Esophageal and gastric dysmotility	[17]
Central nervous system						
Retina						
■Inner plexiform layer	DA	~	✓		Visual disturbances	[38,39]
Olfactory bulb						
■Anterior olfactory n.	ACh, CRF	✓	✓	1	Hyposmia	[17]
Pons		✓	✓			
■Locus ceruleus, raphe and pedonculopontine n.	NA, 5-HT, ACh	✓	✓	2	Sleep disorders, depression, akinesia?	[17]
Midbrain						
■Substantia Nigra	DA	✓	✓	3	Bradykinesia, rigidity, tremor	[17]
Hypothalamus						
■Supraoptic and paraventricular n.	Oxy, VP	~	✓	3		[39,40]
■Tuberomamillary and lateral tuberal n.	HistHyct	✓	~		Hypotension?	[41,42]
■Hypocretin cells (perifornical)		~	✓		Sleep disturbances	[43]
Thalamus						
■Intralaminar n. (centromedian and parafascicular, parataenial)	Glu	✓	✓	3	Motor impairments	[44,45]
Basal forebrain						
■N. basalis of Meynert	ACh	✓	✓	4	Cognitive impairment	[17]
■Amygdala (cortical and basolateral n.)	CCK, Glu	✓	✓	4	Visual hallucinations? Hyposmia?	[46]
■Hippocampus	Glu	✓	✓	4	Mild cognitive impairment	[17]
Cerebral cortex						
■Frontal cortex	Glu	✓	✓	5	Dementia	[17]
■Parietal cortex	Glu	✓	~	6	Dementia	[17]

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); A, adrenaline, ACh, acetylcholine; CRF, corticotrophin-releasing factor; CCK, cholecystokinin;DA, dopamine; Glu, glutamate; Hist, histamine; Hyct, hypocretin; n., nucleus; NA, noradrenaline; Oxy, Oxytocin; VP, vasopressin; ✓, present; ~ , not confirmed or absent.

thin, long and unmyelinated axons [28]. CNS may be accessed through both a nasal and a gastric route via preganglionic fibers of the DMV which innervate the enteric nervous system [47–49]. This hypothesis fits with the neuropathological evidence of LB in the olfactory and enteric systems of both PD and incidental cases [32,50,51] as well as the clinical observations of olfactory deficit and gastrointestinal dysfunction in PD patients, which precede the disease motor onset [52,53].

α-SYN itself may be the pathogenic factor underlying the spread of Lewy pathology throughout the CNS following a prion-like process. This hypothesis has first been proposed after observing that fetal mesencephalic cells grafted into the brain of PD patients 11–22 years earlier contained classical LB inclusions [54–56]. This suggested that α-SYN could be transmitted from the affected host neurons to healthy transplanted neurons, where it recruited normal α-SYN to misfold. Other findings derived from tissue culture and transgenic animals demonstrated cell-to-cell transfer of α-SYN inducing pathological changes and cell death in the recipient [48,57]. Recently, Luk and co-workers demonstrated the widespread propagation of pathological α-SYN aggregates throughout anatomically connected regions of the CNS following brain injection of synthetic α-SYN fibrils into α-SYN transgenic or wild type nontransgenic mice [58]. They suggested a mechanistic link between α-SYN transmission and PD hallmarks as α- SYN pathology resulted in the progressive loss of DA nigral neurons and a consecutive striatal dopamine depletion of sufficient magnitude to induce detectable motor deficits [59].

Accumulating evidence suggests that PD may indeed be a prion-like disorder and that α-SYN behaves like the protein prion (PrP), which underlies disorders such as Creutzfeld–Jakob disease or bovine spongiform encephalopathy. Both proteins share many similarities: (i) they can undergo an aberrant conformational change from a native α-helix to a β-sheet conformation which promotes their self-aggregation, (ii) their protein aggregates can act as "seeds" to recruit and promote the misfolding of wild-type proteins, (iii) their misfolded protein form is recognized to be toxic and induce neurodegeneration [60]. The transmission of LB pathology following a prion-like mechanism through anatomically linked neuronal network might explain the sequential and predictable topographical progression of PD observed by Braak and co-workers. The mechanisms by which intracellular protein aggregates can reach neighboring cells in the CNS are not clear, and may involve neuronal transmission by exocytosis and endocytosis as well as spreading throughout the nervous system via anterograde and retrograde transport.

3. Etiological aspects of PD

Among the many hypotheses surrounding PD etiology, environmental toxin exposure has been the most studied. The awareness of a relationship with PD was raised during the 1980s, when young individuals developed PD signs after an intake of designer drugs contaminated with 1-methyl-4-phenyl-1,2,3,6- tetrahydropyridine (MPTP), a substance similar to the herbicide paraquat [61]. MPTP was then demonstrated to selectively damage nigral neurons by blocking mitochondrial complex I [62]. Since then, many pesticides (i.e., rotenone), herbicides (i.e., paraquat) or insecticides were positively associated to PD risk [63]. Although many environmental, occupational and life-style risk factors were proposed, older age still remains the most significant and well documented one. Conversely, epidemiological data suggest the existence of protective factors such as cigarette smoking and coffee drinking [64], the use of non-steroidal anti- inflammatory (NSAID) drugs [65] or high uric acid levels [66]. As PD prevalence and incidence are lower in women, sex hormones such as estrogens have been suggested to exhibit neuroprotective antioxidant properties [67].

A clear mendelian inheritance can be established in 5–10% of patients. Familial forms constitute a particular category of PD cases often displaying uncommon clinical symptoms – such as young onset or dystonias – and an absence of LB. The first PD mutation was identified in SNCA – the gene encoding α-SYN – in 1997 [68], with additional point mutations, duplications and triplications identified in other kindreds with autosomal dominant PD [69–71]. Interestingly, α-SYN protein turned out to be a major component of LB [72] and SNCA duplications were recently associated to sporadic PD cases [73]. Since then, 6 causative genes have been associated to autosomal dominant (i.e., SNCA, UCHL-1, LRRK2) or autosomal recessive (i.e., Parkin, PINK1, DJ-1) PD and extensively reviewed in [74]. Two novel autosomal dominant genes, VPS35 (PARK17) [75] and EIF41 (PARK18) [76] were recently found in kindreds presenting with late-onset typical PD.

It must be stressed, however, that the vast majority of PD cases are sporadic and may rather be caused by a complex interaction between genetic susceptibility and environmental factors [77]. A few PARK genes such as SNCA [78] or LRRK2 [79], as well as genes involved in other neurodegenerative diseases including MAPT (microtubule associated protein tau) [80] or GBA (glucocerebrosidase) [81] appear to impact PD susceptibility significantly. To date, more than 800 genetic association studies have been published to decipher the missing heritability in PD, often exhibiting inconsistent results [82]. Meta-analyses were recently performed showing genome-wide statistically very significant association of eleven loci *BST1*, *CCDC62/HIP1R*, *DGKQ/GAK*, *GBA*, *LRRK2*, *MAPT*, *MCCC1/LAMP3*, PARK16, *SNCA*, *STK39*, and *SYT11/RAB25* and novel evidence for *ITGA8* polymorphism [82]. However, at the very best, only 60% of the population-attributable risk might be explained by the most promising PD loci identified until now [83].

4. PD research challenges

4.1. Deciphering PD pathogenesis

Despite clues provided by recent genetic breakthroughs and the many alterations observed in the brain of idiopathic PD cases, the molecular mechanisms underlying sporadic PD etiopathogenesis and particularly the massive and selective neurodegeneration in the SN still need to be deciphered. Over the last decades, a variety of neurotoxin-induced and transgenic animals have been constructed to model PD. Although some of these show a massive SN degeneration and a clear PD phenotype, they are less useful to address PD pathogenesis as toxin exposure, for example to pesticides, is by no means a prerequisite for PD to develop. More recently, a variety of transgenic mice have been developed using different strategies, the most common being to incorporate and overexpress mutated genes known to be associated with rare monogenic forms of parkinsonism in humans [84]. So far, however, none of these models has been able to recapitulate all key features of PD [85]. Importantly, most transgenic models have failed to induce significant SN degeneration, LB formation and a clear PD phenotype [86]. In addition, this candidate-based research paradigm is problematic as most PD patients do not exhibit pathogenic gene mutations at the basis of their condition, and, perhaps with the exception of α-SYN, it remains to be established to which extent molecular abnormalities observed in monogenic PD and their animal counterparts are truly relevant to study those underlying sporadic PD.

4.1.1. Pathogenetic mechanisms of PD at a glance

It is generally thought that a combination of environmental factors along with aging initiate a cascade of pathological cellular and molecular events ultimately leading to neuronal demise in genetically susceptible individuals. Many mechanisms have been

shown to sensitize neurons to death but the exact combination and succession of events at work in PD still need to be established. Table 2 summarizes some of the major evidence supporting common hypotheses surrounding PD pathogenesis, which were gathered from recent studies of sporadic and familial PD cases as well as animal models of PD.

Brain deposition of insoluble aggregates containing abnormal proteins, which results in the formation of neuronal intra-cytoplasmic LB in PD, is a hallmark of many neurodegenerative disorders and as such, may underlie a common pathogenic mechanism of neuronal death. Alpha-SYN, whose gene was found mutated in inherited automosal dominant PD cases, seems to play a central role in sporadic PD as it notably turned out to be a major constituent of LB. The mechanisms of aggregation and protein toxicity in PD remain unclear but multiple studies suggest that α-SYN overexpression or misfolding resulting from mutations or post-translational modifications (i.e., nitration, phosphorylation, ubiquitination) may confer toxic properties to the protein and increase its propensity to aggregate [123]. In fact, α-SYN aberrant soluble oligomeric conformations also known as protofibrils might be the more toxic entities. Increasing numbers of aggregation-prone proteins are being identified in LB such as parkin, indicating that α-SYN might not be the only key player. Unraveling the exact composition of LB could provide some clues on other proteins potentially playing a role in PD neurotoxicity.

Under pathological conditions such as proteostatic impairment or during normal aging, the propensity to protein misfolding and aggregation might be enhanced. Several lines of evidence suggest that dysregulation of the main cellular protein degradation pathways – ubiquitin proteasome system (UPS) and autophagy-lysosomal pathways (ALP) – leads to abnormal protein accumulation and cellular toxicity in PD. UPS mediates the selective degradation of

Table 2
Common PD pathogenic hypotheses and their arguments.

PD pathogenic hypothesis	Major evidence		
	Sporadic PD	Familial PD	PD models
Protein aggregation, Lewy Bodies (LB) and α-SYN			
Hypothesis: misfolded or abnormally modified proteins such as α-SYN can accumulate, resulting in LB formation and neurodegeneration. Mechanisms of toxicity are unknown: α-SYN oligomers? LB?	■ α-SYN is a major LB component [72] ■ GWAS association of SNCA [82] ■ SNCA duplications in rare cases [73]	■ SNCA duplication, triplication [69–71] ■ Mutations A53T, A30P, E46K [68]	■ α-SYN mutations, post-translational modifications [87–89], truncation [90] increaseα-SYN aggregation ■ α-SYN deletion confers neurotoxicity resistance [91] ■ α-SYN over-expression in rodents and non-human primates can induce DA neuronal death [92,93] ■ Brain injection of α-SYN fibrils in wt mice induce nigral neurodegeneration and PD-like clinical features [59]
Protein degradation system impairment			
Hypothesis: Dysfunction in main protein degradation systems (UPS and ALP) may lead to protein accumulation, LB formation and neurodegeneration.	■ Both UPS and ALP are decreased in aging brains		■ Both UPS and ALP may degrade α-SYN [94] ■ Pathologic α-SYN can impair UPS and ALP [95,96]
■ Ubiquitin proteasome system (UPS)	■ Altered proteasomal activity in SN, or peripheral blood cells [97,98]	■ Parkin, UCHL-1	■ Proteasome function altered by exposure to toxins (MPTP, rotenone) or high levels of α-SYN [99]
	■ UPS and ubiquitinated proteins in LB		■ Proteasome inhibition induce protein aggregation and neurodegeneration in vitro and in vivo [100,101]
■ Autophagy-lysosomal pathway (ALP)	■ Accumulation of undegraded autophagosomes in nigral DA neurons [102] and alterations of lysosomal markers	■ ATP13A2, LRRK2	■ Loss of autophagy-related gene (atg7) results in protein accumulation and DA neurodegeneration [103]
	■ GWAS association of lysosomal GBA [81]	■ Parkin and PINK-1	■ Parkin and PINK-1 may regulate mitophagy [104]
Mitochondrial dysfunction			
Hypothesis: Mt. dysfunction leads to decreased ATP levels and increased ROS production, which may promote high-energy demanding neuron impairment.	■ Mt complex I activity deficiency (i.e., SN [105], platelets [106], frontal cortex [107])	■ PINK1, Parkin, LRRK2, DJ-1, omi/Htra2	■ Inhibition of mt complex I activity by toxins (i.e., MPTP, rotenone) induce neurotoxicity [108]
	■ Higher amount of mtDNA mutations in DA neurons from PD patients [109]	■ POLG [110,111]	■ Deletion of mttranscription factor TFAM induce DA neurodegeneration [112]
Oxidative stress (OS)			
Hypothesis: OS induce oxidative damage through free radical (NO, ROS) increase and subsequent neuronal death.	■ Alterations in brain iron content, antioxidant defenses (i.e., DJ-1, SOD)) ■ Oxidative damage to lipids, proteins (i.e. α-SYN nitration [87]) and DNA	■ DJ-1	■ Intracellular [113], intra-mt [114], or extracellular [115] free radicals mediate DA neurodegeneration in PD models .
Inflammation/glial reaction			
Hypothesis: Inflammation is involved in self-perpetuating deleterious events that lead to neurodegeneration.	■ Increased pro- inflammatory factors and enzymes (i.e., TNF-α, iNOS, COX2) in PD brains [116–118]		■ α-SYN overexpression induce inflammation [119] ■ Inhibition of microglial activation prevent neuronal loss [120]
	■ Anti-inflammatory drugs (i.e., NSAID) are protective [121]		■ LPS mediated inflammation induce nigral DA neurodegeneration [122]

Abbreviations: COX2, cyclo-oxygenase 2; DA, dopamine; iNOS, inducible nitric oxid synthase; LPS, lipopolysaccharides; MPTP,1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; mt, mitochondrial; OS, oxidative stress; TNF, tumor necrosis factor alpha. Gene names are in *italic*.

short-lived soluble or misfolded proteins tagged with ubiquitin (Ub) chains, through the sequential action of several enzymes (E1, E2, E3). ALP is primarily involved in the degradation of long-lived stable intracellular proteins as well as protein aggregates and organelles [71] via lysosome delivery [124–126] and might constitute a default degradation pathway when UPS is inhibited [127]. Evidence of their impairment in sporadic PD came from the observation of proteasome-related proteins in LB (i.e., ubiquitinated proteins, proteasome components) as well as decreased proteasomal activity and signs of abnormal autophagy in PD brains compared to controls [97,102,128]. Further underlining their importance in PD, they both seem to be involved in α-SYN clearance [94,96]. In addition, recent functional studies demonstrated that many proteins linked to monogenic PD families may be involved in UPS (i.e., E3 ligase Parkin) or autophagy pathways (i.e., lysosomal ATPase ATP13A2, PINK1) [99,129–131]. Interestingly, Parkin, and PINK-1 have been reported to participate in signaling pathways controlling mitophagy [132], an essential mitochondria quality control process whereby damaged mitochondria can be removed. It is however still unclear whether these changes mediate neuronal cell survival or death response.

PD pathogenesis has long been associated to mitochondrial dysfunction and oxidative stress. Mitochondria assume a plethora of essential cellular functions whose alteration might lead to cell demise through ATP energy depletion, increased ROS formation and oxidative stress, or Ca^{2+} homeostasis imbalance. In pathological conditions, a vicious cycle might install whereby damaged mitochondria are in turn a source and a target of ROS, ultimately leading to neuronal loss. Other sources of oxidative stress include DA metabolism, reactive iron deposition, impaired antioxidant pathways or inflammation processes among others. In sporadic PD, their role is notably supported by the reduced mitochondrial complex I activity and increased oxidative levels observed in PD brains [87,133–135]. Substantial insights in the understanding of mitochondrial role and oxidative stress in PD came from the identification of PD-associated genes encoding mitochondrial related proteins PINK1, DJ1, parkin, LRRRK2, α-SYN, or omi/Htra2, whose alterations were shown to affect mitochondrial integrity or increase oxidative damage [108,136]. Recent findings suggest that mutations in the mitochondrial genome (mtDNA), which encodes proteins from the respiratory chain, are also involved in PD pathogenesis.

Inflammation likely contributes to the cascade of events leading to DA neuron death in PD, through mechanisms comprising astrogliosis, microglial activation or lymphocytes infiltration [137]. Epidemiological studies have suggested that inflammatory process can be a risk factor for PD, as regular consumers of non-steroidal anti-inflammatory drugs (NSAIDs) are relatively spared by PD [121,138] and both cellular and molecular post-mortem studies reported inflammatory features in parkinsonian brains [116–118,139–141]. Mechanisms of neurotoxicity may involve the activation of cellular death pathways in DA neurons through the microglia cell release of deleterious pro-inflammatory compounds (i.e., cytokines) or indirectly through the production of microglial-derived free radicals (i.e., NO) [142]. A vicious cycle amplifying neuron destruction referred to as reactive microgliosis could install [143], whereby an acute insult can initiate a self-sustaining inflammatory reaction maintained by a positive feedback from dying neurons [138]. Interestingly, α-SYN aggregates [144] may induce neuronal death through microglial activation as well.

4.1.2. The specific vulnerability of nigral dopaminergic neurons

The selective vulnerability of nigral dopaminergic neurons, which represents less than 0.0001% of all brain neurons, could be attributed to cell-specific risk factors. Briefly, DA has been seen as a culprit, because its metabolism was shown to generate toxic reactive oxygen species (ROS) [145]. However, a variety of non-DA neurons also die in PD and conversely some DA neuron populations are spared arguing against DA as the principal cell-risk factor. Nigral DA neurons, as well as other neurons damaged in PD, have a distinctive impressive axonal field with disproportionally long unmyelinated axonal projections, each of them supporting no less than 370,000 synapses [146]. Comparatively, SN DAergic cell body is small, representing about 1% of the total cell volume [145]. Given their size and complexity, these neurons are associated with an elevated axonal trafficking and a high ATP demand, which might sensitize them to proteostatic stress, aggregation and energetic crisis. This could explain why mutations in genes related to mitochondrial and trafficking activities could predispose to PD. Moreover, adult SN DA neurons have a particular and uncommon physiological phenotype. They are neuronal pacemarkers, exhibiting an autonomous activity in the absence of synaptic input to help maintaining DA levels in the striatum, the main projection target. For that, they rely on relatively rare L-type Ca^{2+} channels Cav1.3, which induce broader action potentials. Contrasting with what occurs in the majority of neurons, those channels are opened frequently with larger magnitude of Ca^{2+} influx [147]. The resulting Ca^{2+} overload could trigger chronic cellular stress and be responsible for SN DA neuron specific vulnerability. Any impairment in Ca^{2+} homeostasis regulation mechanisms such as ATP-dependent pumping as well as mitochondrial and endoplasmic reticulum adequate buffering function might critically compromise SN DA neurons survival. These neurons might additionally exhibit a lower intracellular Ca^{2+} buffering capacity sensitizing them to Ca^{2+} induced stress. They are typically located in regions where calbindin, a Ca^{2+} fast buffering protein, is poorly expressed, whereas neurons in calbindin rich regions are relatively more resistant to death in PD [11,12]. Finally, glutamatergic input to SN could also sensitize them to death through excitotoxicity mediated by NMDA receptor activation [148].

4.2. PD diagnosis and biomarkers

Biomarkers are defined as biological parameters that should be objectively measurable, indicative of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention (Biomarkers Definitions Working Group). Biomarkers research in PD is still in its early stages and only few of the investigated biomarkers have been validated for routine clinical practice yet. PD diagnosis remains largely based on clinical criteria and suffer from several limitations [149]. An early diagnosis was proved particularly challenging due to an overlap of PD symptoms with those of other forms of parkinsonism including multiple system atrophy (MSA), progressive supranuclear palsy (PSP), dementia with LB (DLB) or essential tremor [150,151]. Misdiagnosing was thus frequently observed in the population at a rate of 15% [151]. However when assessed by a movement disorder specialist, PD can be quite accurately diagnosed during a patient's life [152]. Moreover, underdiagnosing rate was estimated at 20% in the population receiving medical attention [151]. Unfortunately, a clinical diagnosis of PD is necessarily postponed to an advanced pathological stage, as about 60% of the nigral dopaminergic neurons are already lost at the time of motor manifestations onset. Currently, a definitive diagnosis of PD can only be made at autopsy, with the neuropathological confirmation of PD hallmarks. Hence sensitive, specific, non-invasive and inexpensive PD biomarkers are needed to (i) detect the disease early or even in preclinical phase and identify at-risk individuals, (ii) provide an accurate and differential diagnosis to distinguish PD from other related syndromes (iii) monitor disease progression and the efficacy of therapy. The following section gives an overview of the most promising biomarkers available.

Recent advances in clinical, neuroimaging or molecular biomarkers have improved the early and differential diagnosis of

PD (Table 3). Olfactory or autonomic function testing – to respectively detect hyposmia [153] or cardiac sympathetic denervation in PD – were developed for an early PD assessment, as nonmotor impairments may precede motor manifestations [154,155]. Functional neuroimaging based on Positron Emission Tomography (PET) or Single Photon Emission Computed Tomography (SPECT) emerged to assess nigrostriatal dopaminergic terminal decline [150,156,157] whereas transcranial ultrasonography allowed the identification of distinct hyperechogenicity patterns in the SN of PD patients [158,159]. While promising these techniques still demonstrate some limitations such as lack of sensitivity, specificity or high costs hampering their widespread use as diagnostic tests. Alternatively, a large number of biochemical compounds (i.e., catecholamines, neuropeptides, amino acids, enzymes, IgGs, oxidative stress proteins) including PD-related proteins (i.e., α-SYN, DJ-1) were typically measured in CSF, blood or urine [160,161] (Table 3). As a major component of LB, α-SYN was one of the most attractive molecules to investigate. In plasma, levels of oligomeric [162] and phosphorylated [163] α-SYN were found increased in PD patients versus controls whereas in CSF, total α-SYN levels [164,165] were found repeatedly decreased, although the increased oligomers/total-α-SYN ratio found in PD might be more valuable [166]. However, conflicting results, significant overlap of values between groups, insufficient sensitivity and specificity preclude the use of α-SYN as a valid marker at the moment [167]. Several studies demonstrated inconsistent results regarding DJ-1 levels in the CSF, whose combination with other molecule measurements might be more helpful for PD diagnosis [168]. Recently, a quantitative Luminex assay demonstrated that the combination of α-SYN and DJ-1 measurements with five other molecules (total tau, phospho-tau, amyloid β_{1-42}, Flt3 ligand and fractalkine) in the CSF could not only help in PD diagnosis and differential diagnosis but was also correlated with disease progression and severity [169]. Given the obvious role of oxidative stress in PD pathogenesis, oxidative markers were investigated. For instance, urinary levels of 8-hydroxydeoxyguanosine were shown to be more elevated in PD versus controls and able to evaluate disease progression [170]. Reduced levels of urate, a strong antioxidant, were found in serum, CSF and in the SN of PD patients, which correlate with DA neurodegeneration, advanced PD symptoms and higher risk for

Table 3
Potential biomarkers for sporadic Parkinson's disease.

Biomarker	Target	Measurement method	Value		Reference
Clinical biomarkers					
Progression on clinical scales	Motor function	Standard clinical criteria (i.e., UKPDSBB, H&Y), timed test (i.e., pegboard)	+: specificity, validated	−: insufficient sensitivity, PD diagnosis at late pathological stage	[149,175]
Olfactory deficit	Olfactory function	Olfactory testing (UPSIT, Sniffin' sticks)	+: early (pre-motor) and differential PD diagnosis, low cost	−: insufficient sensitivity and specificity, correlation with disease duration and severity?	[176,177]
Imaging biomarkers					
Cardiac sympathetic denervation	Autonomic function	^{123}I-MIBG cardiac SPECT	+: differential diagnosis of PD, early PD diagnosis?	−: insufficient sensitivity and specificity, expensive	[178]
Loss of striatal DA or DAT decline	Nigrostriatal pathway function	[^{18}F]-fluorodopa PET/DAT SPECT	+:early PD diagnosis	−: insufficient specificity, modulated by DA drugs, expensive	[179]
Nigralhyperechogenicity	Substantia nigra	Transcranial sonography	+: low cost, non-invasive	−: technically demanding, disease progression?	[180]
Molecular biomarkers					
Levels of: ■total α-syn (↓) ■ratio oligo/ total α–syn (↑) ■DJ-1(↓) ■Urate	CSF	Immunoassay,enzymatic assay	+: non-invasive and promising i.e., urate predictor of PD progession?	−: insufficientsensitivity and specificity , often inconsistent results	[164–166, 168,171]
■Panel of proteins (i.e. DJ-1,α-syn, total tau, phospho- tau, amyloid β1–42, Flt3 ligand, and fractalkaline)	CSF	Immuno-assay (Luminex)	+: good sensitivity and specificity, correlation with disease progression and severity	−: not validated	[169]
Levels of: ■Oligomeric α-syn(↑) ■ Phospho α-syn(↑) ■DJ-1 isoforms ■Urate ■Oxidative stress markers(↑)	Whole blood, serum, plasma or blood cells	Immuno-assay, enzymatic assay	+: not invasive and promising	−: insufficient sensitivity and specificity, often inconsistent results,	[162,163,171, 181–183]
Levels of: ■8-OHdG	Urine	Immuno-assay	+:non-invasive, predictor of disease progression	−: more evidence required	[170]
Levels of: ■ α-syn, DJ-1	Saliva	Immuno-assay	+:non-invasive	−: more evidence required	[184]
Tissue immunoreactivity: ■α–syn	Peripheral tissue biopsy (i.e., colon, skin)	Immunohistochemistry	+:premotor PD detection	−:More evidence required	[32,35,185]

DAT, dopamine transporter; H&Y, Hoehn and Yahr scale; I-MIBG, 123-I- métaiodobenzylguanidine; MSA, multiple system atrophy; PET, positron emission tomography; PSP, progressive supranuclear palsy; SPECT,single photon emission computed tomography; UKPDSBB, UK Parkinson's disease society brain bank criteria; 8-OHdG, 8-hydroxy-désoxyguanosine. Signs "+" and "−" respectively indicate some of the advantages and drawbacks of each biomarker.

developing PD [171–173]. While promising for some of them, none of the above biomarkers – taken individually or in combination – has reached a sufficient level of accuracy and reliability allowing their clinical use [174].

5. Translational proteomics for Parkinson's disease: from benchside to the clinic

The recent emergence of new "candidate-free" unbiased disciplines such as proteomics but also genomics and GWAS, transcriptomics, or metabolomics has boost the exploration of new avenues to decipher molecular pathways at the basis of PD pathogenesis and biomarkers for PD diagnosis. Proteomics is a particularly prominent "omic" discipline which systematically studies the protein complement of cells or tissue at a given time [186]. Around 20,000 human genes produce about 150,000 transcripts and more than 1,000,000 proteins as a results of alternative splice variants, RNA editing or PTMs. At variance with the static genome and limited transcriptome of a particular biological structure, its proteome, which is submitted to constant changes over time, represents a rich, if not infinite, source of information and a unique window into complex molecular regulatory networks, as proteins are often the direct effectors of biological systems. Proteomic analyses have allowed the identification and quantification of thousands of proteins from complex mixtures together with the determination of their modifications (i.e., PTMs) or protein–protein interactions. A typical workflow requires four consecutive steps: sample preparation, protein/peptide separation, mass spectrometry (MS) analysis and finally bioinformatics data processing. The most popular approach, referred to as shotgun or bottom-up, involves the enzymatic digestion of protein samples into peptides. After an overview of the current proteomics methods, we will highlight some of the key proteomic contributions to PD research. Given the current limitations of animal models of PD, which still cannot recapitulate all clinical and neuropathological features associated with sporadic PD [85,187], this section will cover human sample-based proteomic analyses only.

5.1. Proteomic methods

5.1.1. Sample processing prior to mass spectrometry

5.1.4.1. Sample choice and preparation. Because the availability of tissue samples from disease sites is still limited, most proteomic studies have relied on the analysis of autopsy tissues from various brain structures as well as biological fluids such as cerebrospinal fluid (CSF) or blood supposed to reflect the disease state (Fig. 1). CSF is an excellent source of diagnostic biomarker as it is in close proximity to the degenerating brain structures and may thus directly reflect its biochemical state under pathological conditions. CSF collection through lumbar puncture necessitates the intervention of a trained specialist and is not without risk for the patient, which may preclude its use for routine screening. Blood – and its subcomponents plasma, serum and peripheral mononuclear cells – can be easily obtained with very little discomfort for the patient and is expected to reflect pathological brain perturbations through disruption of or passage across the blood–brain barrier. Blood analysis remains challenging given its complexity, as blood proteins are derived from all perfused organs and cell types, its high dynamic range of protein concentrations which may vary by up to 10^{12}, and the presence of a few highly abundant proteins (i.e., 12 proteins) constituting most of the total blood protein content (i.e., 95%) [188]. Urine and saliva have sometimes been used in the field of neurodegenerative disease proteomics. Although they can be easily obtained and collected non-invasively, their analysis is still associated to technical difficulties due notably to their low protein content or high inter- and intra-individual variability.

The preparation of such samples necessitates specific precautions to prevent any analytical bias and allow reproducible comparisons between samples especially regarding their collection, handling and storage [189]. Because CSF protein content is low, blood contamination is a central issue as it may dramatically affect CSF protein levels and lead to conflicting results when measuring biomarker levels such as α-SYN [167]. Storage temperature was shown to affect protein levels as well. For instance, cystatin C was shown to be degraded when CSF was stored at −20 °C but not at

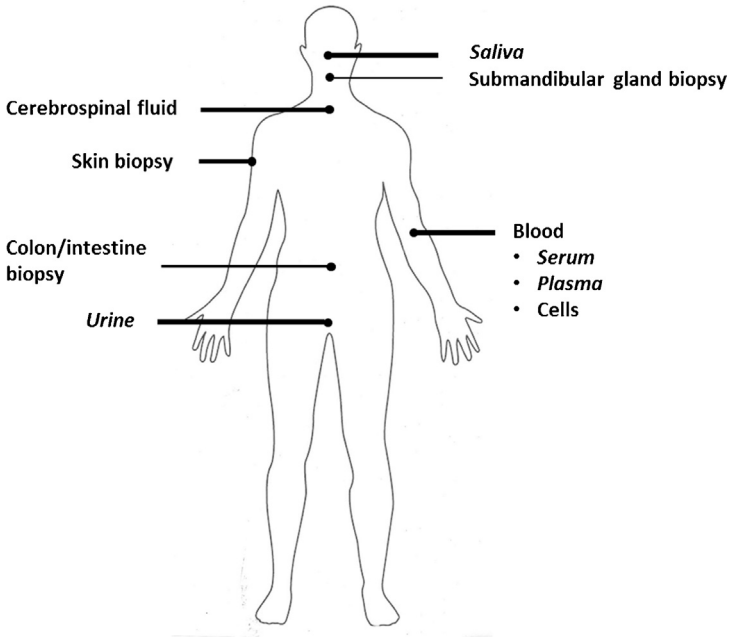

Fig. 1. Potential samples of interest for PD translational proteomics. Line thickness is proportional to sample accessibility, with the thicker line representing the samples collected the more easily (minimally invasive procedure). In italic, samples which currently present the more technical limitations for a proteomics analysis.

−80 °C [190]. When studying autopsy tissues, a particular care must be taken to minimize post-mortem delay (PMD) – the time elapsed between death and sample processing or freezing at −80 °C, ideally under 48 h, at which most protein modifications might occur at room temperature [191].

Efforts are generally placed into sample sub-fractionation at a tissular, cellular or subcellular levels to target the most relevant proteomes. CSF and blood can typically be depleted of their few highest abundant proteins using immunoaffinity columns (i.e., MARS column) to enrich in the many low abundant proteins that could be potential markers of a pathological state. When using autopsy samples, increasing levels of specificity can be assessed with sub-proteome analyses of entire cryo-dissected brain regions such as the cortex [192] or the SN [193–195] down to various sub-cellular fractions of interest such as mitochondria [196], synaptosomes [192], cortical LBs [197,198] or neuromelanin granules [199] .

5.1.4.2. Sample separation. Given a proteome size, dynamics and complexity in biological samples, its complete analysis represents a considerable challenge which it is still not achievable using a single method. Reducing sample complexity prior to MS analysis is therefore an essential step, which requires thought-worthy experimental design. A variety of methods were developed for protein or peptide separation based on their physicochemical properties, either by electrophoresis (i.e., SDS-PAGE, IEF, Offgel), chromatography (i.e., SCX, RP) or immunoaffinity. Multidimensional fractionation can be implemented to enhance proteome coverage and detection sensitivity in MS. Two-dimensional gel electrophoresis (2-DE) is a commonly used gel-based strategy combining IEF and SDS-PAGE, which separates complex protein samples according to their isoelectric point (pI) and molecular weight [200]. A modified form of 2-DE termed difference gel electrophoresis (DiGE) technology, allows sample multiplexing in a single gel using fluorescent dyes [201]. In contrast, gel-free approaches are typically performed using liquid chromatography (LC)-based techniques, which can directly be coupled with MS. Chromatographic techniques involve protein or peptide separation according to their hydrophobicity (i.e., reversed-phase columns), ionic charge (i.e., SCX), size, affinity (i.e., MARS column, IMAC column). Informative subsets of proteins or peptides carrying phosphorylations, glycations, glycosylations or being cysteine-rich can thereby be isolated. Of note, a recently developed technique termed Off- gel (OGE) allows the collection of peptide or protein samples in liquid phase after IEF and is often coupled with LC.

5.1.2. Sample analysis by mass spectrometry

Mass spectrometry (MS) is the core component of proteomic workflows allowing protein analysis through mass over charge (m/z) measurements. Driven by the inextricable complexity of proteomes, technical limits of MS instrumentation are constantly pushed, with the development of multiple ion sources, analyzers or detectors, the three main elements of mass spectrometers. Matrix-assisted laser desorption/ionization (MALDI) [202] and electrospray ionization (ESI) [203] are generally used in proteomics in combination with a variety of mass analyzers including time of flight (TOF), ion trap (IT), quadrupole (Q), Fourier transform ion cyclotron resonance (FTICR) or Orbitrap. Hybrid mass spectrometers enable the determination of protein amino acid sequence, expression level and structural features (i.e., PTM sites) using multiple stage MS fragmentation (MS^n). Ion fragmentation is generally done by collision induced dissociation (CID) but electron transfer dissociation (ETD) may be more suited to analyze PTMs [204]. The ESI linear trap quadrupole (LTQ)-Orbitrap is one of the most performant and recent instrument commercialized, combining the MS^n capability of the LTQ with the high resolution and mass accuracy of the Orbitrap [205–207].

Several bioinformatics tools were developed to interpret MS data. These include tools for peptide/protein identification (i.e., Mascot [208], Phenyx [209]) or PTMs analysis (i.e., Quickmod [210]) based on sequence database search algorithms as well as tools

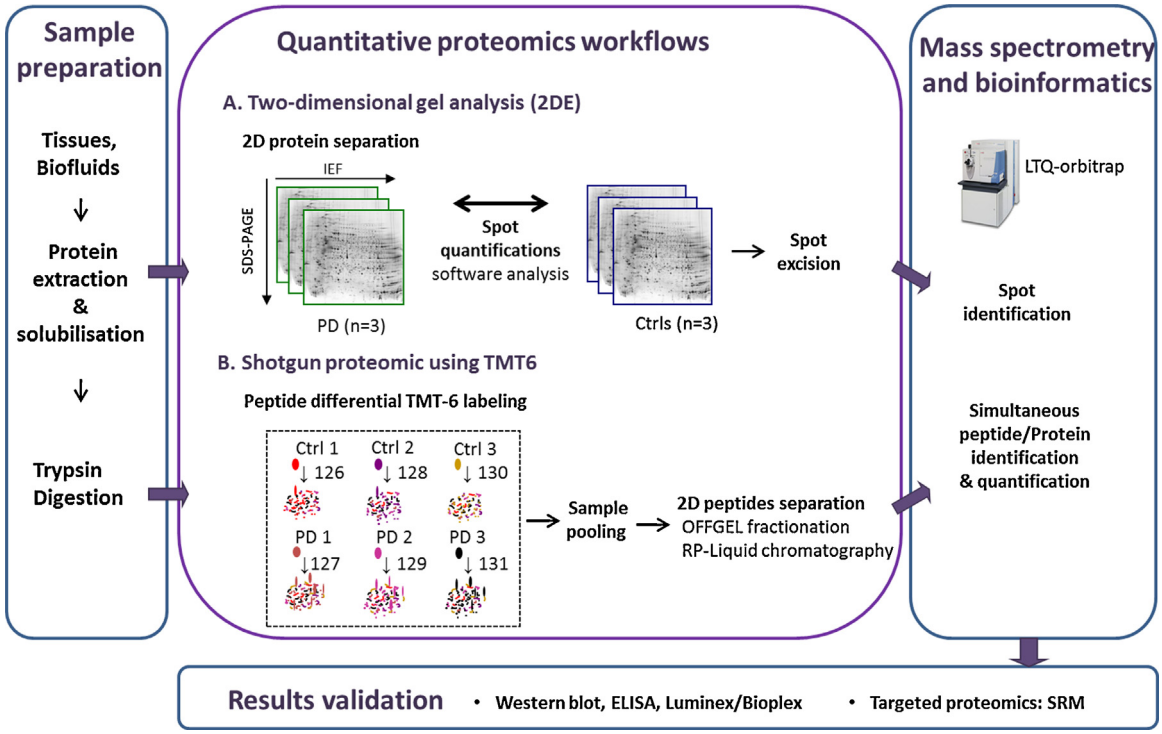

Fig. 2. Quantitative proteomic workflows. Proteins are extracted from biological samples and can either be submitted to a 2-DE workflow (A) or digested and submitted to a shotgun proteomic approach (B). In the 2-DE approach, protein spots are compared between gels using an image analysis software allowing their quantification, and spots of interest are analyzed by tandem MS (MS/MS) for their identification. In the shotgun approach, differentially labeled peptide samples are identified and quantified by MS/MS. Results must be verified by at least one robust orthogonal method.

for protein/peptide quantification (i.e., Isobar, Easyquant [211]). As protein/peptide identification is a probability based process, false discovery rates (FDR) are generally calculated to estimate the rates of mistakenly identified proteins and should generally be kept below 1% at the peptide or protein level [212]. When peptide or protein sequences are absent from databases, often resulting from unexpected PTMs, *de novo* peptide sequencing can be performed manually or using specific programs.

5.1.3. Quantitative proteomics

Quantitative proteomic data are needed to determine the specific set of proteins exhibiting different expression levels in healthy versus pathological states. Relative quantification has traditionally been performed by 2-DE or DIGE, followed by staining and image analysis to identify differences in gel patterns (Fig. 2). Although providing access to a range of PTMs and protein isoforms, the procedure is not best-suited for the rapid analysis of complex samples, suffering principally from a lack of automatization and a limited dynamic range together with reproducibility, resolution and sensitivity issues. Alternatively, high throughput shotgun quantitative proteomic platforms coupled with multidimensional LC has been widely used to tackle complex mixtures, either relying on isotope labeling of proteins or peptides, or "label-free" with quantification based on spectral counting or ion peak intensity [213].While the latter could be more convenient for analyzing large number of samples (ex. clinical screening), it strongly relies on robust sample preparation procedures and may be less reliable for measuring small protein changes [214,215] .

Most proteomic quantitative analyses are thus based on isotope labeling, which consists in the introduction of a mass tag (i.e., heavy or light) to differentiate identical peptides from various samples in MS owing to a mass shift. Isotope labeling can be done at various levels (i.e., cell, protein, peptide) on different reactive groups (i.e., cysteine, lysine containing residues) and allow sample multiplexing. During the past years, several methods were developed including stable isotope metabolic labeling for cultured cells (SILAC), isotope-coded affinity tags (ICAT) or isobaric tagging technologies either using tandem mass tags (TMT) [216,217] or isobaric tags for relative and absolute quantification (iTRAQ) [218,219]. In isobaric labeling, the total mass of the tag is kept constant owing to a mass normalizer group, and identical peptides from different samples sharing the same chromatographic properties co-elute in the mass spectrometer. Labeled peptides thus appear at the same mass in an MS scan, but give rise to low mass reporter signature ions upon CID fragmentation in MS/MS mode (i.e., between 126 and 131 m/z for TMT-6). This robust approach has been one of the most beneficial for the analysis of body fluids and tissues as it allows the simultaneous peptide identification and quantification of up to 10 samples in a single MS/MS experiment. The comprehensive analysis of specific PTMs known to be important for PD, such as oxidation, nitration, phosphorylation, glycosylation or ubiquitination can also be addressed. Generally, proteomes of interest are specifically enriched before being analyzed by MS quantitative techniques. Alternatively, peptides with defined PTMS can be targeted based on their MS fragmentation characteristics (i.e., neutral loss, multiple reaction monitoring MS modes). Selected Reaction Monitoring (SRM) allows the targeting and measurement of selected signature peptides from molecules of interest (reviewed in [220]). Given its unique potential to quantify reliably low abundant analytes in complex mixtures, SRM may represent an alternative to ELISA for clinical validation measurements which are dependent on antibody availability. Importantly, absolute quantification can be obtained through AQUA method, with the spiking of a known quantity of an isotope- labeled peptide as an internal standard, followed by SRM MS analysis [221].

5.2. Translational proteomic

5.2.2. The quest for PD biomarkers

To date, the clinical management of PD patients is still hampered by the lack of reliable diagnostic and therapeutic biomarkers which might pave the way for the development of better options and PD treatment and prevention. Traditional candidate-based studies have assessed the potential of specific targets typically associated to PD pathophysiology as biomarkers of PD, for example the CSF level of α-SYN. In contrast, unbiased biomarker discovery using "omics" technologies such as proteomics has allowed the detection in various human samples of a wide panel of pathological protein expression or PTMs changes, which could serve as novel biomarkers.

In PD, most biomarker discovery studies have relied on the proteome analysis of CSF. Using 2-DE, CSF profiling allowed the detection of a few differential proteins (i.e., complement c3) between control and PD patients [222,223]. Much more changes were detected in the CSF composition of PD patients using shotgun proteomic quantitative strategies as reviewed in [224]. Abdi et al. found 72 proteins – including ceruloplasmin or apolipoprotein H, uniquely associated to PD compared to AD, dementia with LBs and control patient samples differentially labeled with iTRAQ-4plex [218]. Based on these results, Zhang et al. performed a large-scale validation of their best potential candidates using a Luminex assay and found that a panel of eight proteins (i.e., tau, amyloid β-42, β-2 microglobulin, interleukin- 8, vitamin D binding protein, apolipoproteins A-II and E and BDNF) was highly effective at identifying PD [225]. The proteomic analysis of plasma and serum samples was proved challenging considering their complexity and the presence of a few highly abundant proteins. However, recent studies successfully highlighted potential PD biomarkers in blood [226–228], of which the most promising may be plasma apolipoprotein A1 (ApoA1) [227]. This result was confirmed by independent studies based on multiplex and ELISA immunoassays, which suggested that low ApoA1 levels correlated with early PD onset and greater dopaminergic deficit as measured by putaminal DA transporter binding [229]. Alternatively, peripheral blood lymphocytes were investigated, highlighting a panel of five proteins (cofilin, tropomyosin, gamma-fibrinogen, ATP synthase beta and basic actin variant), which may be useful for PD diagnosis [230].

In the future, other sources of potential biomarkers accessible *in vivo* may be investigated by proteomics (Table 1, Table 3). Moreover, as shown on Fig. 1, tissue biomarkers may be found in peripheral regions susceptible to Lewy pathology such as submandibulary gland, colon, or skin [50,53,185,231]. These regions could be accessed through biopsy in living patient and could allow the detection of early disease biomarkers, as the peripheral nervous system may be involved before the central nervous system in PD. Saliva was recently analyzed given its connection to the submandibular gland, which produces most of the salivary volume [29]. Importantly, α-SYN and DJ-1 were successfully identified in saliva, providing further relevance for the study of this fluid in a biomarker context [184]. Finally, unbiased proteomics investigations of post-mortem tissues from selected PD-relevant brain regions of neuropathologically confirmed cases might provide useful candidate biomarker proteins, which could further be screened in biofluids using immunoassay or targeted proteomics such as SRM. This approach is expected to provide highly specific PD markers given its direct access into PD pathological sites in neuropathologically confirmed PD cases.

5.2.3. Improving PD pathogenesis understanding

Effective therapeutic interventions directly impacting PD biology are urgently needed to slow, interrupt or ideally reverse the inexorable progression of the neurodegenerative process. Advances in the understanding of the specific pathological actors mediating

molecular events at the basis of neurodegeneration in PD may open new avenues for treatment and perhaps prevention of the disease. Although helpful, hypothesis-driven or "candidate-based" approaches might have reached some limits in the understanding of PD pathology, overwhelmed by the impressive complexity and diversity of the processes likely engaged in PD. In the last 10 years, unbiased proteomic studies have been undertaken in human PD-relevant brain regions to gain new insights into PD pathogenesis. Autopsy tissues were generally used, allowing the analysis, in neuropathologically confirmed cases, of the key brain structures selectively affected in PD, which are not accessible to *in vivo* biopsy. Although taken at a late pathological stage, these samples may provide a unique window into the specific abnormalities occurring in PD brains in the absence of any validated PD animal model. However, only a small number of studies have been published as yet due to the scarcity of human tissue samples available.

5.2.4. Characterizing brain proteomes

Proteomic profiling of PD-relevant brain regions has generated the identification of extensive protein datasets, whose characterization has helped to understand their specific functions within the CNS and their particular vulnerability in PD. In a recent shotgun proteomics study, our group established the more comprehensive catalog of nigral proteins with 1795 identifications in PD and control patients [232]. The GO analyses suggested a critical involvement of high energetic supply, anti-oxidant defense, cytoskeletal organization and vesicular transport in SN function. As PD lesions extend towards cortical regions at advanced disease stages, the proteome of frontal cortex was characterized, leading to 812 protein identifications in cytosolic, mitochondrial, synaptosomal or nuclear fractions. Many of those proteins appeared to be involved in neurodegenerative diseases [233]. To dig deeper into the brain proteome, the content of subcellular fractions were examined. Leverenz et al. managed to analyze 2′ 500 cortical LB isolated by laser capture microdissection from patients with dementia with LB disease, discovering 296 proteins [197]. Although a few proteins were validated by IHC localization, future investigations may exclude contamination from the surrounding tissues. Another group used a sucrose gradient centrifugation strategy to enrich cortical LB from LB variant of AD, yielding to the identification of 40 proteins which were not present in a negative control [198]. Altogether, these studies may help to decipher the mechanisms of LB formation and the pathways leading to neurodegeneration in PD. Tribl et al. carried out the first proteomic profile of intact neuromelanin (NM) granules enriched from control human SN using density gradient centrifugation [199]. Seventy-two proteins were identified, of which many were closely linked to lysosome-related organelles [199]. Of note, the protocol has been recently improved to allow the combined enrichment of neuromelanin and synaptosomal fractions using far less starting material (<0.15 g) [234]. This important development may allow collecting a sufficient amount of NM from PD patient nigral tissues, which are severely depleted in NM-containing cells. A link between NM and PD pathogenesis was hypothesized as NM-containing neurons seem to be more vulnerable in PD [235]. Moreover, NM interacts with iron, which is known to accumulate in the parkinsonian SN. Recently, a targeted proteomic approach revealed that L-ferritin was an NM granule component, providing new clues on iron storage mechanisms in the NM-containing neurons [236]. These investigations provided insights into NM composition, mechanisms and function, which are still poorly characterized, and may help to understand iron-driven degeneration of the SN in PD.

5.2.5. Identifying proteome alterations in PD brains

To gain more insights into the disease pathogenesis, quantitative proteomic data may allow the complex proteome alterations occurring in the brains of PD versus control patients to be disentangled. 2-DE studies of human brain tissues targeting the SN were conducted, highlighting several abnormalities in the

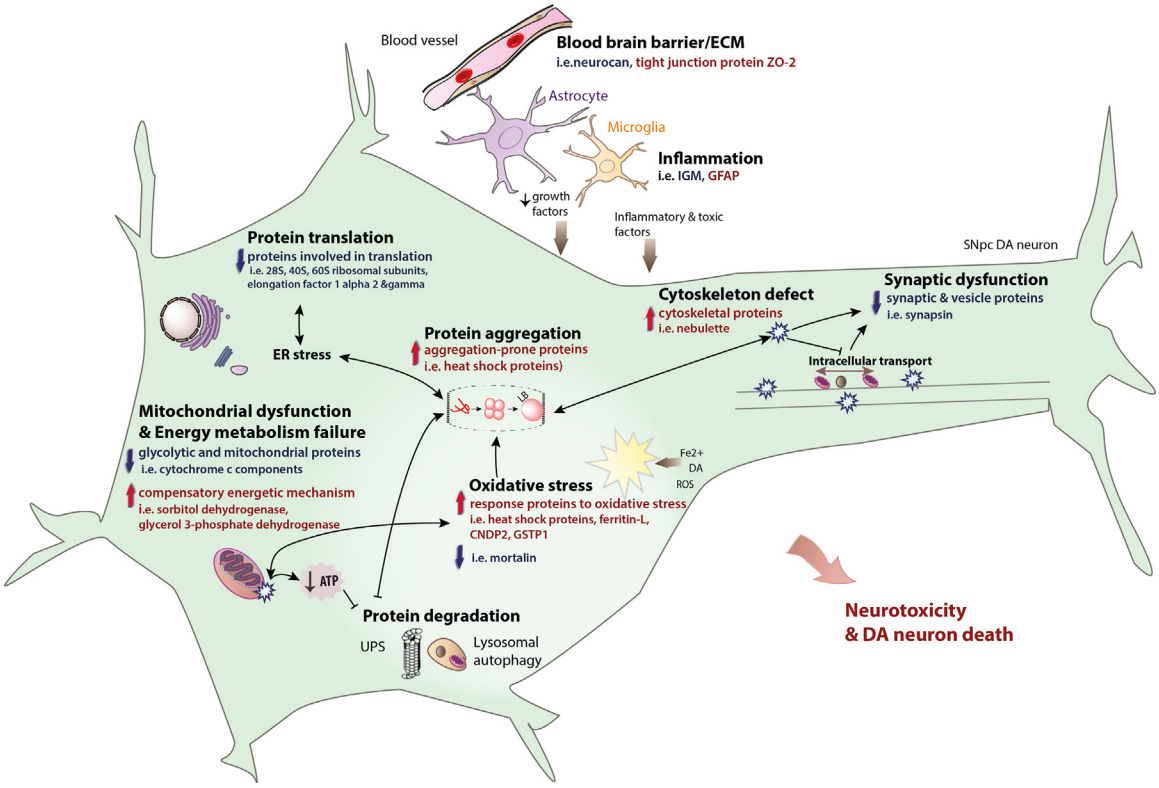

Fig. 3. Proteomics confirmed existing hypotheses surrounding PD pathogenesis and highlighted less conventional ones (image modified from [232]). Red and blue colors represent proteins found respectively over- and under-expressed in PD.

proteome of PD patients [152,153,192]. For example, our group was able to identify CNDP2 or VPS29 overexpression in PD. Using a shotgun approach combined to ICAT, others found 119 proteins exhibit changes in their relative expression in mitochondrial fractions obtained from the SNpc of PD cases compared to controls [196]. Of these, mortalin decrease in PD was confirmed using a cellular PD model and functional biology experiments suggested a major role for mortalin in PD neurotoxicity through mechanisms that may involve oxidative stress, mitochondrial and proteasomal dysfunction [196]. Taking advantage of the sixplex TMT tagging technology to compare the nigral proteome of PD patients ($n = 3$) versus controls ($n = 3$), our group observed significant expression level changes in 204 proteins. PD-relevant candidates were further characterized including nebulette, whose overexpression might be associated to neurodegeneration in PD through mechanisms that may involve disruption of cytoskeletal dynamics [232].

A few proteomic comparative studies have focused on post-mortem cortical tissues. Two studies using iTRAQ labeling to profile frontal cortex samples of PD patients at different stage of the disease and control cases, suggested a potential association of respectively mortalin and glutathione-S transferase Pi (GSTP1) with disease progression [192,237]. In the first study, mortalin underexpression was observed in the cytosolic fraction of PD patients and validated by independent methods [237]. In the second study, GSTP1 overexpression was observed in the synaptosomal fraction of PD cases and was suggested to protect cells against rotenone-induced neurotoxicity via oxidative and ER stress attenuation in a PD cell model [152]. Three other studies by Choi et al. proved useful for elucidating some of the PTMs associated with PD. Using 2-DE, they demonstrated oxidation in multiple proteins previously linked to PD, including the chaperone DJ-1, superoxide dismutase Cu/Zn, as well as the de-ubiquitinating protein UCH-L1 in the frontal cortex of PD patients compared to controls [238–240]. Recently, van Dijk et al. performed a proteomic analysis of the locus ceruleus, one of the earliest affected brain regions in PD [241]. By comparing PD patients ($n = 6$) versus controls ($n = 6$) with a label free approach, they identified 2′ 495 proteins of which 87 were differentially expressed between groups. In particular, a pathogenic role for aminoacyl-tRNA-biosynthesis was highlighted.

Overall, these proteomics studies were successful in confirming existing theories about PD pathogenesis (Fig. 3). The majority of the differential proteins were indeed implicated in mitochondrial dysfunction, energy metabolism impairment, oxidative stress, protein aggregation, cytoskeleton impairment, or inflammation. Whereas some of the observed protein alterations were previously associated to PD pathogenesis (i.e., ferritin), others were novel candidates such as CNDP2, mortalin, regucalcin, or seipin. Curiously, α-SYN overexpression did generally not show up significantly in these studies [196,232,241]. The most probable explanation comes from the fact that in a tissue-based approach, the overexpression of synaptic α-SYN in surviving DA neuronal PD cells may be compensated by the higher number of healthy neuronal cells in control patients. These studies also suggested some less conventional pathways such as defects in protein translation, ER stress, blood brain barrier or extracellular matrix abnormalities (Fig. 3). Of note, it was sometimes unclear whether the observed protein changes were a cause or a consequence of the neurodegenerative process. In tissue-based approach, the decrease in neuronal protein levels may simply reflect PD associated neuronal loss. Further biological evaluation of the pathogenic mechanisms underlying these protein alterations may provide new therapeutic targets for PD.

5.2.6. Some perspectives

During the past 10 years, only a small number of human tissue based proteomics studies have been published due to limitations in their availability, number, quality and complexity. In the context of a worldwide decline in autopsy rate, some of these issues can be partially overcome through a facilitated access to existing brain banks which ensure the collection of well characterized and preserved brain tissues. There are probably over a hundred brain banks worldwide, an incomplete list of which can be accessed at the BrainNet Europe website (http://www.brainnet-europe.org). Moreover, a novel technology might allow the molecular imprinting of basal ganglia tissues obtained during deep brain stimulation (DBS) from living PD patients. Taking advantage of the temporary access to specific target regions during the implantation of DBS electrodes for PD treatment, the approach may allow the capture of small tissue amounts (i.e., about 20 μg of proteins) using a chemically modified micro silicon chip placed at the tip of the surgical dilator, as demonstrated in monkeys [242]. If applicable to humans, the use of *in vivo* brain tissue imprints would reduce PMD to a few minutes avoiding protein degradation and may allow the observation of changes occurring early in PD course, although control samples might be more difficult to obtain for comparisons. Finally, the great complexity and cellular heterogeneity characterizing human brain regions may be further addressed by additional cellular and subcellular fractionation steps. In the SN, mixed cell populations together with the characteristic neuronal DA loss in PD may have obscured the identification and quantification of subtle changes limited to DA neurons. Laser- capture microdissection (LCM) together with the emergence of more sensitive MS techniques and automated methods to collect cells offer now the possibility to specifically isolate and investigate separately small defined areas including neurons, facilitating data interpretation. The selective dissection of DA neurons neurons by LCM might allow to dig deeper in the DA neuron proteome and to reveal the specific pathological mechanisms responsible for their demise in PD.

5.2.7. Concluding remarks

Somewhat disappointingly, comparative proteomic studies have received little attention from the neuroscience community yet. This might be due to several factors including the absence of well- defined hypotheses and the low concordance rates observed between studies. It is generally difficult to compare proteomic studies together, as many sources of variability can drastically influence the final outcome. First, samples themselves are greatly heterogeneous, as a consequence of patient's history, co-morbidities, PD subtype, disease duration or therapies, all hardly controllable parameters. Tissue quality can also affect protein changes, when PMD delays are too long or different between groups. Second, the lack of standardized protocols for sample handling, preparation (i.e., dissection, solubilization buffers) and analysis may prevent inter-laboratory comparisons as well. In fact, the plethora of existing analytical methods may lead to variability in the identified proteome. This translates into small overlaps in protein identifications across proteomic studies. For example, more than 1200 nigral proteins of our recently identified dataset were not identified in the few other proteomic investigations studying SN [193,195,196]. Third, the generally small number of samples examined by proteomics is associated with a low statistical power for detecting differentially expressed proteins. The lack of stringent criteria to select differential proteins together with the absence of further result validation, an almost impossible task given the usually large differential protein datasets, may lead to the identification of false positives and false negative candidates. Consequently, only a small percentage of proteins were found similarly differential across the few proteomic studies analyzing SN for instance, although a high proportion of non-differential proteins were concordant between them. The fact that differential proteins are sometimes found inversely expressed across studies may indicate the presence of different protein

isoforms that may still participate in the same pathogenic mechanism. Indeed, PD is known to be a heterogeneous disease and distinct alterations in common pathways may induce a common phenotype. For example, different point mutations and multiplications of α-SYN all result in familial PD. Similarly to what is generally thought for transcriptomic data, the absence of concordance between proteomic studies could be due to the utilization of protein list for comparisons, rather than standardized pathways, which could indicate the involvement of common pathogenic mechanisms. To conclude, instead of being taken as conflictual, results obtained in proteomics may rather be seen globally, each proteomic study identifying a fraction of the changes occurring in the SN and contributing step-by- step to a better knowledge of the extraordinarily complex molecular jigsaw puzzle at the basis of PD.

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