

Blood and cerebrospinal fluid markers in Parkinson's disease: current biomarker findings

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Abstract: Parkinson's disease (PD) is a common, progressive, and disabling neurodegenerative movement disorder. Signs and symptoms begin insidiously and overlap with other neurodegenerative conditions, making diagnosis challenging in early disease. Moreover, there are as yet no established biomarkers that will objectively measure disease progression, and this hampers efforts to obtain neuroprotective therapies. At present, diagnosis, measurement of progression, and response to therapeutic intervention rely almost exclusively upon clinical observation. There remains, therefore, a critical need for validated biomarkers. Recent advances in understanding the underlying pathophysiology of PD have aided in identifying strong candidate markers to assist in diagnosis and evaluate progression. Pathways that are disrupted in PD include protein misfolding, mitochondrial dysfunction and increased oxidative stress, dysregulated inflammatory processes, and altered gene expression. For example, α -synuclein is a key contributor to PD pathology, in which misfolded and damaged α -synuclein accumulates in neurons; this is now a leading candidate as a PD biomarker in cerebrospinal fluid. There is also recent evidence that markers of Alzheimer's disease may be "repurposed" to provide information on PD diagnosis, prognosis, and nonmotor symptoms, including cognitive dysfunction. At the same time, increasingly sophisticated bioinformatics technology allows an unbiased approach to biomarker identification, for example, using proteomic, transcriptomic, and metabolomic analysis. Such generally accessible blood or cerebrospinal fluid tests, if successful, will likely lead to significant improvements in PD diagnosis, management, and research.

Keywords: Parkinson's disease, neurodegeneration, α -synuclein, oxidative stress, tau

Background

Parkinson's disease (PD) is a common, progressive, and disabling neurodegenerative disease affecting approximately 1% of people over 65 years of age. In the US, the combined direct and indirect costs of PD amount to approximately \$25 billion per year. Worldwide prevalence is predicted to at least double from 2005 to 2030¹ and this will lead to increasing challenges to optimal health care. The cardinal clinical signs of PD are bradykinesia, rigidity, and rest tremor, affecting speech, hand coordination, gait, and balance. These are attributed for the most part to progressive degeneration of dopaminergic input from the substantia nigra within the midbrain to the striatum,² thus disrupting complex downstream pathways critical for motor function. Molecular and chemical pathways involving dopamine have, therefore, been a priority as targets in biomarker development, with considerable success in initial studies using neuroimaging of the dopamine pathway. However, it is increasingly understood that PD pathology is

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more widespread, involving multiple complex biochemical, molecular, and cellular pathways, that exist both within the central nervous system as well as systemically.

Objective biomarkers for PD would serve to advance clinical care and research in several different ways. A first critical need for a PD biomarker is in diagnosis. PD diagnosis is still made upon clinical grounds, combining history and physical findings. Unfortunately, clinical diagnosis can be challenging, and PD presents insidiously and heterogeneously. Multiple studies have underlined the risk for diagnostic error.^{3,4} This is a particular risk in early disease when symptoms and signs are subtle and may overlap with other neurological disorders.⁵ A valid diagnostic biomarker for PD would not only improve diagnostic timeliness and accuracy, but would potentially define disease subtypes.⁶

A second critical need is to meet the challenge of measuring progression of the underlying neurodegenerative disease. Changes in disease status, for example in response to treatment or with disease progression over time, are still measured using clinical rating scales, in particular the Unified Parkinson's Disease Rating Scale (UPDRS).⁷ However, signs and symptoms are at least partially masked by symptomatic treatment, thus confounding clinical rating scale evaluations of PD progression. This has likely contributed to recent frustration over the difficulty translating promising preclinical and early phase clinical trial neuroprotection data into approved therapies. Hence, there is optimism that a biomarker incorporated as a clinical trial outcome measure, or "surrogate marker", would objectively and quantitatively track PD progression, opening the way to more sophisticated testing of potential neuroprotectants.

A third critical need is the ability to determine "target engagement" for any given intervention. This would be informative in making go/no go decisions during early-phase drug development. It would also flesh out understanding of clinical trial results. For example, in the case of an unsuccessful clinical trial, if target engagement could be verified, it would prompt examination of whether the target itself is worth pursuing in drug development.

Biomarker development in PD has traditionally been focused on neuroimaging, which therefore holds a significant lead time. However, there is an emerging focus upon blood and cerebrospinal fluid (CSF)-based markers.⁸ These have a number of advantages, including accessibility, cost, greater variety of targets available for testing, and a generally lower burden on the patient. The availability of increasingly sophisticated technology has now opened up the possibilities for biofluid-based

biomarker development. Moreover, breakthroughs in understanding the neuroscience and molecular genetics of PD have supported development of targeted approaches to biomarker development. This article describes the most relevant recent studies in biomarker development using CSF and blood components as biosamples, and examines potential future directions.

Developing landscape of CSF and blood biomarkers for PD

Advances in understanding the molecular and genetic underpinnings of PD have identified "key players" that may be targeted as potential biomarkers (Figure 1). These are components of pathways that lead to important processes in PD etiopathogenesis, including disrupted dopamine metabolism, aberrant protein aggregation, oxidative stress and mitochondrial dysfunction, inflammation, and transcriptional dysregulation. Moreover, the "omics" technologies have raised the possibility of unbiased identification of potential biomarkers. The following is not an exhaustive list of potential markers, but describes progress in identifying promising avenues based upon both targeted and untargeted approaches (for summary, see Table 1).

Dopamine-based biomarkers

Although dopamine deficiency in the nigrostriatal pathway is a primary feature of PD, and it would therefore seem intuitive that dopamine levels in biofluids might be altered, measuring dopamine directly in blood and CSF has proved disappointing. Older studies examined endogenous

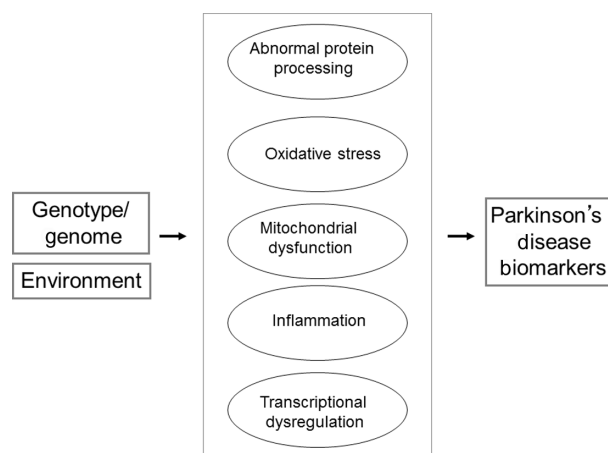


Figure 1 The central panel lists cellular pathways disrupted in the pathogenesis of PD that have been targeted for development of PD biomarkers. These abnormalities are thought to arise from a combination of genetic and environmental factors, as depicted on the left side of the panel.

Abbreviation: PD, Parkinson's disease.

Table I Promising Parkinson's disease-associated biomarkers in blood and CSF

	Blood	CSF	Comments
Proteins and peptides			
α -synuclein (PARK1 locus)	Variable	↓	May correlate with worsened motor disability
	↑ antibodies		Specific isoforms likely important
DJ-1 (PARK7 locus)	Variable	↓	May depend on specific isoform
A β_{1-42}		↓	May correlate with cognition
p-tau		↓	May associate with postural instability-gait disorder subtype
t-tau		↓	May associate with worsened motor disability and cognition; rate of change may correlate with progression
ApoA1		↓	
IL-10, RANTES	↑		
TNF- α	↑	↑	
Antineuronal antibodies, antmelanin antibodies		↑	
RNAs: transcriptome changes			
			Proteasome subunit- α type-2, laminin β -2, aldehyde dehydrogenase I family-member A1, histone cluster-I H3e; ST13; heat shock 70 kDa protein 8, p19 S-phase kinase-associated protein 1A, huntingtin-interacting protein-2, 19S proteasomal protein PSMC4, aldehyde dehydrogenase family I subfamily A1
miRNAs			
			↓ miR-339-5p, miR-30c, miR-148b, ↑ miR-223, miR-324-3p, miR-24; ↓ miR-29a, miR-29c, miR-19a, miR-19b
Lipid-based			
Malondialdehyde, F(2)-isoprostanes, cholesterol oxidation products	↑		Reflects oxidative damage
Small molecules			
HVA		↑ xanthine/HVA	May associate with PD progression
8OHdG	↑	↑	Reflects oxidative DNA damage
Urate	↑	↑	May associate with progression
Hypoxanthine, hypoxanthine/xanthine ratio, and xanthosine/xanthine ratio	↓		

Abbreviations: A β_{1-42} , β amyloid 42 amino acid isoform; ApoA1, apolipoprotein A1; CRP, C-reactive protein; CSF, cerebrospinal fluid; HVA, homovanillic acid; IL-6, interleukin-6; IL-10, interleukin 10; MCP-1, monocyte chemoattractant protein 1; miRNAs, micro RNAs; p-tau, phosphorylated tau; RANTES, Regulated on Activation, Normal T cell Expressed and Secreted chemokine; t-tau, total tau; TNF- α , tumor necrosis factor alpha; 8OHdG, 8-hydroxy-2-deoxyguanosine.

tetrahydroquinolones reflecting dopamine metabolism, and salsolinol was suggested in one study to be associated with PD dementia.⁹ Altered levels of dopamine metabolites and related compounds have been more recently demonstrated. Homovanillic acid (HVA) is a product of dopamine breakdown. A recent study employing metabolomic analysis compared levels of the purine compounds, xanthine and HVA. The CSF ratio of xanthine/HVA was demonstrated to differ significantly between 217 patients with PD and 26 controls, with a higher value in PD compared with controls.¹⁰ PD participants were not taking anti-PD medications, thus the difference could not be attributed to medication effects. Moreover, the xanthine/HVA ratio increased with time in a longitudinal element of the study, suggesting that it is a possible marker of both state and trait. Not only is there disruption of dopamine production in PD, but there is also evidence that its metabolism may be disrupted. In particular, the enzyme responsible for

dopamine breakdown, monoamine oxidase B, has been found to be increased in plasma samples collected from patients with PD.¹¹

α -synuclein, a key component in PD pathogenesis

Protein damage and misfolding are key to PD pathogenesis. These processes are important in formation of the structures that comprise the hallmark pathology of PD, ie, Lewy bodies (Figure 2). The α -synuclein gene was the first locus identified in which specific mutations result in Mendelian inheritance of PD (PARK1 gene locus), and although these mutations are rare, identification of α -synuclein protein as a contributor to PD pathogenesis revolutionized understanding of this process. Importantly, these pathological changes may occur in the “premotor” phase of PD, therefore also making α -synuclein a strong candidate for monitoring PD both before and after motor symptoms arise.

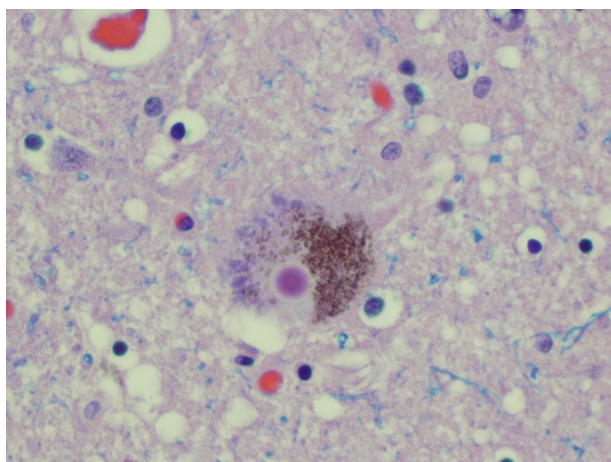


Figure 2 Photomicrograph of a hematoxylin-eosin-stained section taken from the substantia nigra pars compacta at autopsy of an individual with PD. A dopaminergic neuron in the center of the field contains a densely stained cytoplasmic Lewy body. **Note:** This article was published in *Handbook of the Neuroscience of Aging*. 2009, Schwarz C, Henchcliffe C. Parkinsonian syndromes. In: Hof P, Mobbs C, Editors. 441–447. Copyright ©2009 Academic Press.²

α -synuclein is primarily intracellular, but is detectable in CSF¹² and plasma,^{13–15} and decreased CSF α -synuclein levels in PD have now been demonstrated in a number of studies.^{12,16–23} Two of these studies examined patients with early PD, making it unlikely that results are simply associated with anti-PD medication effects.^{22,23}

These studies have raised hopes that α -synuclein could be a first diagnostic marker for PD. However, specificity remains to be better defined. One study found decreased CSF α -synuclein in Alzheimer's disease (AD) versus controls, leading the investigators to postulate that it is a marker of loss of synapses in general.¹⁶ Similarly, decreased CSF α -synuclein has been seen in dementia with Lewy bodies and multiple system atrophy.²¹ Whether CSF α -synuclein levels will objectively track PD progression remains to be determined, but there are some hopeful preliminary data. The Parkinson's Progression Markers Initiative (PPMI, ClinicalTrials.gov identifier NCT01141023) enrolled 400 participants with early PD, and baseline data showed that a decreased CSF α -synuclein concentration was associated with increased motor severity in PD.²² This will now need to be tested longitudinally. Interestingly, in a post hoc analysis of CSF α -synuclein levels in the DATATOP (Deprenyl And Tocopherol Antioxidative Therapy Of Parkinsonism) study, lower levels were associated with cognitive decline longitudinally.²⁴

An important issue to consider is whether a combination of protein measures incorporating α -synuclein might constitute a superior biomarker. In one study, a panel of seven proteins comprising α -synuclein, DJ-1, total tau (t-tau), phosphorylated tau (p-tau), fractalkine, Flt3 ligand, and

$A\beta_{1-42}$ differed in CSF samples from PD when compared with normal controls, AD, and multiple system atrophy.²⁵

Although blood is more accessible than CSF in the clinic, developing blood-based measures of α -synuclein has proven more complicated. In plasma, total²⁶ and oligomeric¹³ α -synuclein has been increased in PD, but this has not been replicated in other studies.^{15,27,28} It has been proposed that variations in sample collection and possibly hemolysis might lead to variability in measures, as the vast majority of α -synuclein is present in red blood cells.

Finally, anti- α -synuclein antibodies have been detected in association with PD, and are worthy of consideration as potential markers of disease. These specific antibodies have been detected in one study in approximately 50% of individuals with sporadic PD, 90% of individuals with familial PD, and 30% of control cases.²⁹ Although another study failed to confirm the finding,³⁰ a recent study examined autoantibodies to the monomeric form α -synuclein and found an increase in these antibodies in serum in PD.³¹

There is, consequently, a need for further studies to better delineate the potential role for α -synuclein as a PD biomarker. It may be that the specific forms of α -synuclein need to be investigated individually, for example, the ratio of nitrated to non-nitrated α -synuclein as suggested by Sharma et al,⁸ to make sense of the variability in findings to date.

Markers of oxidative stress and mitochondrial dysfunction

Mitochondrial dysfunction has been widely documented in PD, with defects in oxidative phosphorylation, calcium homeostasis, and biogenesis.^{32,33} Early studies determined deficits in individual complexes in the electron transport chain, for example, mitochondrial complex I in platelets.³⁴ Moreover, Charnoly bodies, abnormally aggregated mitochondrial membranes, are found in degenerating neurons. It has been suggested that these structures as well as components affecting their formation (such as metallothioneins) could serve as PD biomarkers.⁸ Increased oxidative stress, with damage to DNA, RNA, proteins, and lipids, are closely linked since mitochondria are the major cellular sources of damaging free radicals. Markers that would reflect key changes in these interrelated processes have therefore been of great interest. Altered redox status in PD has been demonstrated in studies of two potent antioxidants, coenzyme Q10 in blood^{35,36} and CSF³⁷ and glutathione in plasma samples.³⁸ Oxidative damage to DNA has been demonstrated in PD by detection of elevated levels of 8-hydroxy-2-deoxyguanosine,

a compound formed by direct oxidative damage to nucleosides in CSF, plasma, and urine.^{39,40} Markers of oxidative damage to lipids are similarly elevated. Malondialdehyde, formed as reactive oxygen species degrade polyunsaturated lipids, was found to be significantly increased in plasma levels in PD,^{38,41} and plasma F(2)-isoprostanes (a fatty acid peroxidation product) and cholesterol oxidation products have been found to be elevated in PD.⁴²

Urate is included in this section since it is a potent antioxidant, as well as a metal chelator capable of binding iron.^{43–45} It is the end product of purine breakdown, with precursors including hypoxanthine and xanthine, which have also been examined as possible biomarkers of PD. Multiple studies have found the urate concentration in serum and CSF to be inversely associated with occurrence of PD, particularly in men.^{46,47} Moreover, higher serum and CSF urate concentrations have been associated with slower PD progression.^{47,48} Hypoxanthine, the hypoxanthine/xanthine ratio, and the xanthosine/xanthine ratio were also found to be reduced in a study of PD versus controls.⁴⁹ As noted earlier, the xanthine/HVA ratio is perturbed in PD and changed with disease progression in one study.¹⁰ This deserves further study in longitudinal cohorts to verify the potential of this ratio as a potential marker of progression.

Markers of disrupted inflammatory pathways

There are multiple lines of evidence demonstrating the presence of inflammation in PD. Microglial activation is observed in autopsy specimens of brain tissue in PD, and neuroimaging using the peripheral benzodiazepine receptor ligand PK11195 is consistent with microglial activation in living PD patients.³³ It is therefore not surprising that several studies have now described alterations in inflammatory pathways in blood and CSF in PD. Increased levels of interleukin-10 and the chemokine RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) have been found in serum collected from individuals with PD compared with controls.⁵⁰ Elevated tumor necrosis factor (TNF)- α levels have been detected in association with PD in both plasma and CSF.^{51,52} Abnormal antibody expression associated with PD includes CSF antineuronal antibodies,⁵³ anti- α -synuclein antibodies,^{29,31} and antimelanin antibodies.⁵⁴

Although these examples are associated with PD diagnosed on the basis of motor symptoms, a proinflammatory state seems to be associated with certain nonmotor symptoms of PD.⁵⁵ Worse scores on psychometric testing have been associated with increased soluble TNF receptor levels

in plasma.⁵⁶ Measures of CSF markers, including C-reactive protein, interleukin 6, TNF- α , interferon 10, monocyte chemotactic protein 1, and macrophage inflammatory protein 1- β , in individuals with PD found inflammatory markers to be significantly associated with more severe depression, anxiety, fatigue, and cognition.⁵⁷ These independent lines of evidence therefore support inflammation as an important pathway in PD, in which perturbations might serve as biomarkers with different purposes.

PD genetics and biomarker development

Major advances in understanding the genetic underpinnings of PD have identified a number of proteins that are key in cellular pathways that are altered. As noted earlier, α -synuclein is now a leading target for development as a PD biomarker. However, other PD-associated genes are now under intensive investigation as biomarker candidates.

One such gene is *DJ-1* (PARK7 locus). Mutations in the *DJ-1* gene are a rare cause of autosomal recessive forms of PD, and the corresponding protein is detected in both blood and CSF. As with α -synuclein, in blood the overwhelming majority of the protein is contained within blood cells and platelets, making sample preparation demanding, and with variable hemolysis or residual platelets in samples leading to a potential source of noise.²⁷ This may account for contradictory results published in plasma.^{58,59} CSF measures may therefore turn out to be more robust. A recent study of DJ-1 protein described decreased levels in CSF samples from study participants with PD compared with healthy volunteers. With findings of 90% sensitivity and 70% specificity, performance characteristics were encouraging.¹⁷ Moreover, in this cohort, CSF α -synuclein and DJ-1 levels correlated. Once again, however, findings have been contradicted by other groups: a previous study found increased rather than decreased CSF DJ-1 levels associated with a PD diagnosis, particularly in early stages.⁶⁰ Moreover, although levels may be associated with the diagnosis of PD, DJ-1 does not seem on the basis of current studies to be a marker of disease severity.^{25,27} As with α -synuclein, however, DJ-1 exists in multiple forms. It will likely be important to define which will be most helpful as biomarkers. In a study of seven DJ-1 isoforms detected in blood, those with post-translational 4-hydroxy-2-nonenal modifications were altered in late PD.⁶¹ In an independent study, DJ-1 oxidized on cysteine-106 specifically was found to be elevated in red blood cells in PD.⁶² Initial results are therefore somewhat encouraging, with the caveat that careful consideration of

the various isoforms of DJ-1, including post-translational modifications, will be necessary.

AD biomarkers and PD: β -amyloid and tau

β -amyloid ($A\beta$) and tau proteins are being intensively studied in AD, and measures of the 42 amino acid isoform of β -amyloid ($A\beta_{1-42}$) and t-tau and p-tau concentrations in CSF are now included in AD diagnostic criteria. There is also evidence that they predict AD risk.⁶³ However, based upon strong recent evidence, these proteins may turn out to be at least as important for use as PD biomarkers. In ventricular CSF from postmortem PD cases compared with controls, it was found that the p-tau/ $A\beta_{1-42}$ ratio and apolipoprotein A1 levels were significantly associated with the diagnosis of PD.⁶⁴

The potential for development of amyloid and tau proteins as PD biomarkers in CSF is further supported by several independent studies. The PPMI is an international longitudinal observational study following 400 subjects with early PD who are drug-naïve, and healthy matched control subjects are also enrolled for comparison.⁶⁵ In this PPMI cohort, lower $A\beta_{1-42}$ and p-tau CSF levels were significantly associated with PD.²² Moreover, decreased t-tau levels were found to be associated with increased severity of motor symptoms. Interestingly, lower $A\beta_{1-42}$ and p-tau were both associated with the subset of PD subjects with postural instability-gait disorder rather than those categorized as having tremor-predominant PD. Since tremor predominance in symptom pattern has been associated with better outcome than postural instability-gait disorder in multiple independent studies, this raises the possibility that lower CSF levels of $A\beta_{1-42}$ and p-tau proteins might be predictive of a worse prognosis.

The DATATOP study⁶⁶ was designed to test whether deprenyl (selegiline) and/or α -tocopherol would slow PD progression, but has also proved invaluable for data mining, given that clinical data, blood samples, and CSF were collected in this large PD cohort over many years. Recent analysis of 403 CSF samples from the DATATOP study found that $A\beta_{1-42}$ levels had a weak negative correlation with total UPDRS scores in early PD, and baseline p-tau/t-tau and p-tau/ $A\beta_{1-42}$ ratios were found to have a negative correlation with progression as measured by the rate of change of UPDRS scores.⁶⁷ A significant (although weak) positive correlation was also observed between disease progression as measured by change in UPDRS score and the rate of change in t-tau level and t-tau/ $A\beta_{1-42}$ ratio. As in AD, these proteins may

also prove to be markers that reflect cognitive decline. CSF levels of $A\beta_{1-42}$ and t-tau proteins, as well as $A\beta_{1-42}$ /total tau ratio, have been described to correlate with multiple scores of cognition, including Logical Memory (delayed), Category Fluency, Digit Symbol, and Trails B minus A, in 22 non-demented individuals with PD.⁶⁸

Finally, combinations of $A\beta_{1-42}$ measures with other proteins may also prove worthy of further investigation. The CSF complement 3/ $A\beta_{1-42}$ ratio and factor H/ $A\beta_{1-42}$ ratio⁶⁹ have both been found to be associated with PD motor severity, cognitive impairment, and dementia. The fractalkine/ $A\beta_{1-42}$ ratio in a cross-sectional study of PD CSF samples correlated with disease severity, and also with disease progression in samples collected longitudinally in this study cohort.²⁵ As in AD, reduced $A\beta_{1-42}$ levels have been associated with cognitive decline in CSF samples from PD patients.⁷⁰

Omics technology and PD biomarker discovery

In contrast with the targeted markers discussed above, the omics approach has been applied to PD biomarker development for unbiased and systematic evaluation of patterns of variation in RNAs (transcriptomics), proteins (proteomics), lipids (lipidomics), or small molecules (metabolomics). Omics combines experimentation and mathematical modeling, making use of large datasets acquired from pools of biological molecules that may be present in blood, CSF, urine, or tissue.⁷¹ Use of powerful mass spectrometry, nuclear magnetic resonance, and related techniques has been central to developing this field. For example, mass spectrometry is capable of high sensitivity detection and of quantifying thousands of molecular species.⁷² The approach provides the possibility to define combinations or “fingerprints” of molecular species that may serve as disease biomarkers. Although early in application in PD, omics analyses have already demonstrated promise in neurodegenerative disorders,⁷³ and it is hoped that identification of one or more species (protein, peptide, RNA, or small molecule), or a composite “fingerprint” will prove feasible that could be of use in diagnosis, measuring disease progression, or determining a response to a specific intervention.

Multiple studies employing transcriptomic analysis have demonstrated dysregulated gene expression in PD brain tissue.^{74–76} In some cases, such differences have served as a basis for identification of potential biomarkers in blood. For example, based upon differential expression in brain tissue, 12 selected candidate transcripts were subjected to analysis using quantitative reverse transcription polymerase chain

reaction (RT-PCR) in blood samples.⁷⁷ The investigators demonstrated that a measure comprising transcript levels for proteasome subunit- α type-2, laminin β -2, aldehyde dehydrogenase 1 family-member A1, and histone cluster-1 H3e, was capable of achieving over 80% sensitivity and specificity for association with the diagnosis of PD. In a case-control study of 66 subjects, a combination of eight gene transcripts were associated with risk of PD, and results were subsequently validated in an independent sample set.⁷⁸ It is intriguing that one of these transcripts encodes the co-chaperone ST13, a stabilizer of heat shock protein 70, which is known to modify α -synuclein misfolding and toxicity. Other transcripts have been identified by omics analysis as potential candidates for PD biomarkers. Five transcripts were found to be altered in blood in early PD, encoding the following proteins: heat shock 70 kDa protein 8; p19 S-phase kinase-associated protein 1A; huntingtin-interacting protein-2; 19S proteasomal protein PSMC4; and aldehyde dehydrogenase family 1 subfamily A1.⁷⁹ As predictors of PD diagnosis, the panel had high sensitivity and specificity.

There is growing interest in proteomic analysis to identify PD biomarkers. In an initial study of human substantia nigra pars compacta tissue, two-dimensional gel electrophoresis identified nine specific proteins that were differentially expressed in PD when compared with healthy control subjects,⁸⁰ and a further analysis increased this number, identifying 221 differentially expressed proteins.⁸¹ Moreover, a rationale for altered expression in PD for many of these proteins can be postulated. For example, differential expression of glutathione S-transferase and other genes involved in glutathione metabolism might relate to the importance of oxidative stress in PD pathogenesis. Altered expression of glial and related proteins, such as GFAP, GMFB, galectin-1, and sorcin, might reflect known changes in activity and presence of glial cells as PD pathology accrues. The challenge of translating this technology to fluid-based biosamples has now been met. Using a different proteomic platform, over 1,500 CSF proteins were analyzed using isobaric Tagging for Relative and Absolute Protein Quantification (iTRAQ) followed by mass spectrometry.⁸² Samples were compared between PD (based upon clinical diagnosis), dementia with Lewy bodies (autopsy confirmed), AD (autopsy confirmed), and healthy controls. Of the 1,500 proteins, 72 were differentially expressed in PD, and specific combinations of proteins were identified that distinguished the different groups. A study of CSF proteins compared surface-enhanced laser desorption/ionization time-of-flight mass spectrometry in 56 subjects with PD, 24 control subjects, as well as samples from individuals with

the clinically closely related disorders of multiple system atrophy, progressive supranuclear palsy, and corticobasal degeneration. CSF levels of ubiquitin, beta2-microglobulin, and two secretogranin 1 fragments differentiated PD from atypical parkinsonism,⁸³ although they did not differentiate PD from controls. Using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS), other protein species have been identified that are differentially expressed between PD and controls,^{84–86} and recent analysis of proteins captured by magnetic bead-based weak cation exchange followed by MALDI-TOF-MS in a blinded analysis identified a combination of five biomarkers discriminating PD and healthy controls with 85% sensitivity and 70% specificity.⁸⁷ Finally, use of two-dimensional gel electrophoresis and mass spectrometry has so far yielded encouraging data supporting the possibility of developing a diagnostic biomarker on the basis of a proteomic fingerprint.^{88,89}

The potential for defining fingerprints of combinations of lipids is now being explored. A recent investigation of lipids isolated from blood samples of healthy adults, with cognition measured in the normal range, determined a set of ten lipid species predicting development of amnesic mild cognitive impairment or AD.⁹⁰ Since overlapping cellular processes, including effects upon cell membrane integrity, may be at play in PD, this approach will need to be thoroughly evaluated.

Metabolomic profiling, which examines arrays of low molecular weight species, has also proved promising in identifying PD biomarkers. In plasma, metabolomic analysis using high-performance liquid chromatography with electrochemical coulometric array detection for quantitative assessment of approximately 2,000 small analytes has successfully distinguished subjects with PD versus healthy controls.³⁹ In plasma samples from 15 drug-naïve PD patients and 25 controls, metabolomic analysis was capable of deriving fingerprints that completely distinguished the two groups ($P < 0.01$). From these fingerprints, it was then possible to choose a subset of analytes that had the most significant contribution to differences measured. This same analyte subset was shown to be capable of distinguishing individuals with PD treated with carbidopa/levodopa versus controls, as well as individuals with PD treated with both carbidopa/levodopa and dopamine agonists versus controls ($P < 0.01$). Using the same platform for metabolomic analysis, in a further study of individuals with idiopathic PD and *LRRK2*-associated PD, these two groups could be distinguished from each other, and also from controls.⁴⁹ It is encouraging that when examining levels of individual analytes, urate (see the

“Markers of oxidative stress and mitochondrial dysfunction” section) was found to be lower in idiopathic and *LRRK2* PD than control samples.^{39,49} A smaller decrease was measured in *LRRK2* nonmanifesting carriers, and, although not statistically significant, it suggests that urate needs to be further investigated in “at risk” individuals.⁴⁹ Additionally, measures of dopamine and purine metabolites (hypoxanthine, HVA/xanthine, xanthosine/xanthine) have been demonstrated to be significantly reduced in PD subjects not taking anti-PD medication when compared with controls.⁴⁹ As noted earlier, use of a metabolomics analysis platform also detected abnormalities in the HVA/xanthine concentration ratio in PD that changed with disease progression over a 2-year period.^{10,91}

MicroRNA profiling

MicroRNAs (miRNAs) are small noncoding RNAs, and are thought to play a pivotal role in regulating gene expression at the post-transcriptional level, affecting transcript stability and translation of multiple genes, including *α-synuclein*. A number of studies have demonstrated differences in specific miRNAs of brain tissue in PD versus healthy controls, but more recently key differences have been detected in circulating miRNAs, making their profiling in peripheral blood a promising approach for biomarker development. Vallelunga et al recently reported a set of nine specific miRNAs out of approximately 750 examined that could distinguish PD from controls and subjects with multiple system atrophy.⁹² A non-overlapping set of four specific miRNAs was found in an independent study to be downregulated in PD, including the genetic *LRRK2*-associated form of PD, versus controls.⁹³

Conclusion and future directions

There is a critical need for validated biomarkers in the clinic and in PD research. The use of fluid-based biomarkers has many advantages over neuroimaging. Testing blood or CSF often places less of a burden on the patient, is easier to perform and therefore not limited to selected medical facilities, and is usually cheaper. Moreover, the use of fluid-based biomarkers provides access to a greater variety of molecular processes that may be at play in PD. At this time, obtaining biomarkers based upon blood or CSF remains in the early phase of development for PD. Many of the findings described in this review still require validation. However, there are now a small number of candidate fluid-based markers that are ready for incorporation into testing of target engagement, such as CSF urate level after administration of inosine.⁹⁴ Early biomarker studies described in this review have focused largely on cross-sectional studies comparing PD with healthy or disease controls, and this will identify potential

markers for diagnosis. However, longitudinal studies will be paramount to identify markers of disease progression. It will also be important to define how early in the disease process any identified diagnostic marker will be useful. There is now hope to identify PD in its premotor phase, and testing fluid-based biomarkers in at-risk cohorts will be critical. It may be that a biomarker useful in the early stages of PD will be different than one useful in advanced PD, and it is therefore doubtful that a single biomarker will suffice in a disorder as complex as PD.

With rapid development of biomarker technologies, the availability of resources for discovery and validation is now vital. As noted above, the longitudinal observational PPMI study is an outstanding example of an international collaborative initiative that is collecting biosamples in early PD, as well as clinical and neuroimaging data, under rigorous conditions.⁶⁵ The BioFIND study (Fox Investigation for New Discovery of Biomarkers in Parkinson's disease, ClinicalTrials.gov identifier NCT01705327) is ongoing at the time of writing, and will similarly provide a platform for discovery and validation of biomarkers in a well-defined cohort of individuals with moderate-to-advanced PD as well as control subjects for comparison.⁹⁵

Development of blood-based and CSF-based biomarkers is now a high priority. Validated biomarkers hold the promise of improving diagnostic accuracy, accurately measuring disease progression, and objectively evaluating the molecular, biochemical, or neurophysiological impact of a given intervention. Ultimately, the hope is that biomarkers discovered in studies of PD will be applicable in premotor PD, ie, to facilitate development of neuroprotective and/or neuropreventive interventions in at-risk populations of patients who have not yet manifested motor symptoms of PD.

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