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Biochemical Pre-motor Biomarkers for Parkinson Disease

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Abstract

A biomarker is a biological characteristic that is objectively measured and evaluated as an indicator of normal biological or pathologic processes or of pharmacologic responses to a therapeutic intervention. We review the current status on target protein biomarkers (e.g. total/oligomeric α -synuclein and DJ-1) in cerebrospinal fluid, as well as on unbiased processes that can be used to discover novel biomarkers. We also give details about strategies towards potential populations/models and technologies, including the need for standardized sampling techniques, to pursue the identification of new biochemical markers in the pre-motor stage of Parkinson disease in the future.

Keywords

Biomarkers; genomics; metabolomics; Parkinson disease; pre-motor; proteomics

1) Parkinson disease, pre-clinical diagnosis and biochemical biomarkers

The precise mechanisms underlying Parkinson Disease (PD) remain unclear and therefore require a better mechanistic understanding to ultimately identify marker candidates that are closely associated with the specific disease process (proximal markers). More than 50% of dopaminergic (DAergic) neurons in the substantia nigra pars compacta (SNpc) are degenerated when motor symptoms of parkinsonism is apparent, which, while enabling the clinical diagnosis of PD according to current clinical criteria¹, makes it exceedingly challenging for any existing and/or future protective/regenerative therapies. It has been noted recently that many non-motor symptoms precede the motor symptoms of parkinsonism² and should therefore be considered earlier during the disease course of PD. Biomarkers detecting PD in this “pre-motor phase” are urgently needed for neuroprotective therapies. The most mature biomarkers currently used to support the diagnosis of PD are derived from functional neuroimaging that assesses the integrity of the nigrostriatal system with ligands specific for dopamine (DA) metabolism or transport. However, because functional neuroimaging is expensive and not widely available, it is not suitable for routine clinical use. Also, it remains to be determined whether neuroimaging can reliably assess PD progression or treatment effects. Furthermore, at it stands now, these techniques are not suitable to differentiate between PD and other disorders with parkinsonism, such as multiple system atrophy (MSA), progressive supranuclear palsy (PSP) and others. Finally, current neuroimaging techniques only reflect changes in the integrity of DAergic terminals but are not indicative of the underlying processes leading to progressive neuronal loss.

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Consequently, additional markers, particularly the biochemical markers reflecting PD pathogenesis at early stages of the disease, are needed to enable specific PD diagnosis, to follow PD progression, and to assess possible effects of interventions. It should be stressed, however, in the discovery of biochemical biomarkers, as of today, functional neuroimaging is the “gold-standard” used to define patients/subjects unequivocally at pre-motor stages.

Another major issue that needs to be emphasized is the heterogeneity of PD. Currently, the only mentioned clinical biomarker in the official criteria is the response to DAergic treatment, but many other PD clinical phenotypes to be discussed later are not treatable by DA medicines. Additionally, a peripheral component of PD is increasingly appreciated recently^{3,4}; therefore the simultaneous measurement of several biomarkers for PD by so-called multiplexing is highly desired, and consequently heavily emphasized in the review.

2) Strategies to identify pre-motor PD biomarkers

It is impractical to screen general population for pre-motor biomarkers. In our opinion, there are a few potential ways of facilitating biomarker discovery in pre-motor stages.

First, clinical testing of those with recognized risks for developing PD

It is known that some non-motor features precede the onset of motor symptoms of PD for years. While these pre-motor signs are non-specific, with limited sensitivity/specificity for their clinical utility², it is anticipated that objective pre-motor biomarkers can be identified in enriched populations based on the known non-motor features with high conversion rates for PD. Such studies could enclose participants with olfactory deficits, constipation, rapid eye movement (REM) sleep behavior disorder (RBD), and depression.

Second, following asymptomatic subjects with genetic mutations that give rise to familial PD

At least in theory, pre-motor PD markers can be searched for in familial PD (fPD) patients owing to genetic mutations, e.g. α -synuclein, DJ-1, pink1 and LRRK2. LRRK2 subjects are one of the ideal populations for pre-motor PD marker discovery because: 1) they avoid ambiguity of the markers discovered in animal models; 2) LRRK2 mutations produce parkinsonism closely resembling idiopathic or sporadic PD (sPD); and 3) unlike other PD genes, LRRK2 mutations are relatively common (1–2%) in typical late-onset sPD, thus providing adequate numbers of patients (and pre-motor relatives) for study of biomarkers. Nonetheless, there are also downsides to this approach in initial biomarker discovery. For instance, it is impossible to obtain the SNpc or striatum from these patients at early stages to study tissue specific markers, greatly decreasing the possibility of identifying tissue specific early stage biomarkers by “-omics” technologies (see below for more discussion).

Third, using animal models

Identifying markers in animal models initially, whether toxicant or genetic-based, and then testing these markers in human PD could be another effective way of discovering pre-motor biomarkers. One of the more effective animal models is nonhuman primates treated with N-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). This model is not only the most investigated PD model available to date but also allows for identification of tissue/disease-specific markers related to nigrostriatal degeneration. The obvious caveat associated with the MPTP model, including the chronic progressive one used by several groups⁵ or any other animal models, such as the rotenone model and others (reviewed by Dehay et al recently⁶), centers around whether or not it faithfully replicates human PD.

Thus, practically, one integrated approach could be using all three models in parallel to minimize the limitations associated with each model discussed above. Also, because advanced proteomics allows multiplex quantitative analysis, it is possible to compare the protein markers in monkeys and LRRK2-PD patients along with sPD cases, thereby enhancing the ability to identify the markers relevant to sPD. These markers can also be tested in those with non-motor symptoms to further enrich the populations at risk before expensive imaging methods are brought in to confirm the diagnosis of pre-motor stages. The hypothesis of this design is that, despite the fact that each model likely has its own unique aspects (and limitations), nigrostriatal neurodegeneration and associated cellular responses are common features to all of these models (shaded area in Fig 1), meaning that there will be a subset of markers common to all models.

3) Biochemical biomarkers

Since brain-derived proteins rarely appear in peripheral biological fluids, such as blood and urine, optimal laboratory markers for neurodegenerative disease processes may not always be easily accessible. On the other hand, the collection of cerebrospinal fluid (CSF) is minimally invasive and very well-tolerated⁷, especially in older adults with a neurodegenerative illness. Furthermore, wider usage of new instruments, such as an 'atraumatic' needle for CSF collection, has decreased the incidence of side effects, such as a transient, positional headache that was often caused by a needle-induced tear in the arachnoid lining around the central nervous system (CNS)⁸. Additional evidence to support human CSF as an ideal source for identifying biomarkers for neurodegenerative diseases, including PD, is comprised of: 1) CSF is most proximal to the site of pathology; 2) CSF reflects the metabolic state of the brain under varying conditions and CNS diseases affect protein concentrations and patterns in CSF considerably; and 3) while 80% of the CSF proteins derive from the blood, 20% of the CSF proteome are synthesized within and released by resident cells of the CNS by means of active secretion, normal attrition, cell injury, or neural death^{9,10}. Indeed, CSF obtained from PD patients modifies survival of DA neurons in a mesencephalic culture differently from those of controls^{11,12}. In addition, analysis of human CSF with silver-stained twodimensional gel electrophoresis (2-D gel) has demonstrated that expression of some proteins may be linked to PD specifically¹³. Another important consideration is that, as discussed earlier, CSF is readily available, which makes longitudinal molecular analyses of changes in CSF during the course of neurological diseases possible. In fact, many groups are actively investigating biomarkers in CSF, and many candidate markers have been revealed. In the next few sections, we will summarize major results obtained in symptomatic PD patients as well as discuss the potential ways of going forward with premotor biomarker discovery.

3a) Targeted markers, α -synuclein and DJ-1, two proteins intimately associated with PD pathogenesis

Total and oligomeric α -synuclein—Predominantly expressed in the synaptic neuronal terminals, 140 amino-acid-long α -synuclein has been found to be the major constituent of the intracellular aggregates in Lewy bodies, the pathological hallmark of PD and dementia with Lewy-bodies (DLB), and in the glial cytoplasmic inclusions of MSA^{14,15}.

Intracellular α -synuclein has been shown to be released into the extracellular space¹⁶, thereby promoting neuron-to-neuron transmission¹⁷. Full-length α -synuclein has been detected in biological fluids, including plasma, conditioned cell media and most recently saliva^{16,18}. The quantification of extracellular α -synuclein has been proposed as a potential biomarker for synuclein-related diseases¹⁹⁻²¹. Discrepant findings by several investigators using several different platforms and operating procedures remain challenging, but the majority of investigators show a reduction of CSF total α -synuclein in the synuclein-related

disorders, including PD, DLB and MSA - a consensus is emerging. In addition, commercially available assays are on their way, and quantification methods of the total α -synuclein or mono- vs oligomeric forms are also being developed.

The mechanism of the decreased CSF α -synuclein in Lewy body diseases, nonetheless, remains unclear to date and could result from various scenarios, such as the reduction of the α -synuclein release into the extracellular space due to intracellular aggregation; an alteration in SNCA gene transcription²², mRNA splicing²³, or protein processing²⁴; a higher CSF flow with lower permeation of plasma α -synuclein into CSF; an enhanced clearance rate of α -synuclein from CSF²⁵; or yet unidentified factors or any combination of mechanisms¹⁹. While only one investigator so far showed a direct (negative) correlation with CSF α -synuclein levels and severity stages of PD in a cross sectional study comprising of 33 PD patients²⁶, very little is known about 1) its impact in the early (drug-naïve or even pre-motor) phase of synuclein-related disorders, 2) its potential value as progression marker shown in longitudinal cohorts, or 3) its significance regarding correlation with different clinical phenotypes in PD. Last, but not least, possible medication effect through DAergic therapy needs to be ruled out and possible relevant post-translationally modified (PTM) α -synuclein species need to be investigated¹⁹.

As mentioned earlier, assays to determine total or mono- vs oligomeric forms of α -synuclein have been established recently based on the same monoclonal antibody for capture and detection²⁷. It appears that CSF values of oligomeric α -synuclein make up to 10% of the total α -synuclein content. Additional investigations show an increase of CSF oligomeric α -synuclein in PD compared to patients with Alzheimer's disease (AD), PSP and controls^{27,28}. Together with the reduced CSF total α -synuclein, the ratio of oligomeric/total α -synuclein reveal a sensitivity of 89.3% and specificity of 90.6% for the diagnosis of PD in one study²⁸.

DJ-1—DJ-1, a multifunctional protein involved in many cellular processes, including the response to oxidative stress, has been linked to PD in those with autosomal recessive gene mutation of the encoding region (familial PARK7)²⁹ as well as in sporadic forms^{30,31}. DJ-1 is present in CSF and showed elevated levels in PD, especially in the early stages (Hoehn and Yahr stages I-II) compared to advanced stages (Hoehn and Yahr stages III-IV) and healthy controls³¹. Recently, however, decreased levels of DJ-1 were found in a large cohort of CSF samples of PD patients compared to healthy controls and AD patients³².

Since elevated levels of oxidized DJ-1 have been found in the brains of PD and AD patients³⁰, specific antibodies recognizing C106-oxidized DJ-1 have been developed and await validation in biological fluids.

However, as discussed earlier, one of the major limitations of these ongoing investigations relates to the fact that PD patients, even at a clinically “early stage”, have already lost >50% of their nigral DAergic neurons at the time of diagnosis and all investigated cohorts so far were more advanced³³. As a result, it is unclear whether these markers can be used for preclinical or pre-motor diagnosis of PD or monitoring of PD progression at early stages. Overcoming this limitation requires performing biomarker discovery in subjects at the asymptomatic stage, which is impractical unless one can first identify individuals at high-risk for developing PD as discussed above.

The other major challenge to the field is variation and sometimes even contradictory results of different investigators. Further investigations of the specificity of the antibodies and the total and oligomeric α -synuclein enzyme-linked immunoabsorbent assay (ELISA) techniques are needed. Independent studies of CSF α -synuclein are also necessary in optimally processed pre-motor cohorts of CSF as well as in other biological fluids. Finally,

future investigations with larger and longitudinally characterized cohorts are needed to clarify a potential correlation and the possible use as progression markers or markers of pre-motor stages.

It should also be mentioned that, in addition to α -synuclein and DJ-1, other targeted biomarkers have been linked to an elevated or decreased risk of developing PD. Of particular note is the observation that the neurodegeneration in PD is lower in the subjects with high urate levels, which is an antioxidant, and in fact urate has been proposed as a neuroprotectant (please see a review by Cipriani et al³⁴ for details). Other key proteins, e.g. parkin, and ubiquitin involved in PD pathogenesis could also be investigated as potential PD markers.

3b) Unbiased markers by "-omics" techniques

In addition to targeted approaches, discovering novel biomarkers unique to PD, symptomatic or pre-motor, can be greatly facilitated by “-omics” techniques, e.g. transcriptomics, proteomics, and metabolomics, which have been developed in the last decade. Each technique has been applied to human samples (brain tissue, CSF, blood, plasma, urine) and reported a set of analytes with potential to differentiate PD from controls. In the next section, we will discuss a few markers revealed by each platform, along with potential caveats and shortcomings. Theoretically, these cutting-edge technologies can be applied to models/enriched populations (Fig. 1) for discovering pre-clinical markers.

Transcriptomics—The use of brain tissue in microarray analysis has provided invaluable insights to PD pathogenesis, including regional specificity, i.e. DA neurons in the SNpc are more sensitive (than those in the ventral tegmental area or VTA) to neurodegeneration^{35,36}. However, the use of peripheral biological tissue sources, such as blood, where mRNAs can also be isolated readily, allows for a minimally invasive means of sampling a population and provides the researcher the ability to perform a longitudinal assessment of particular markers of interest in order to give an indication of pathways that may be altered as the disease progresses³⁷. In this regard, by comparing blood taken from control and early stage PD patients, Scherzer and colleagues^{38,39} have identified ST13, a gene that was highly under-expressed in PD samples compared with control samples. ST13 is a cofactor for heat shock protein-70 (Hsp70), a protein that plays critical roles in the misfolding⁴⁰ of modulating protein, e.g. α -synuclein. Unfortunately, a follow-up investigation by another group did not replicate this observation⁴¹.

Several important caveats that deserve consideration when undertaking a transcriptomic-based evaluation of mRNA expression differences between disease and normal states include: 1) not all alternations in mRNAs translate into changed protein or metabolite levels; 2) it remains controversial as to whether gene alterations in peripheral samples, i.e. WBCs obtained from blood, represent what is happening in the CNS. For the latter point, it should be stressed that PD is not just a disease with CNS pathology only (also see discussion on pre-motor symptoms above).

Proteomics—Like transcriptomics, proteomics generates enormous amounts of data, both identification and quantification of proteins, when comparing one disease state to another. On the other hand, in contrast to transcriptomics, in which all genes analyzed can be quantified simultaneously, identification of proteins by current proteomic technologies are heavily dependent upon abundance of proteins and pre-processing of samples (see a previous review⁴² for more details on this topic).

A few attempts have been made to reveal novel markers in CSF for symptomatic PD⁴³⁻⁴⁵; however, independent investigations are needed to validate these candidate markers, e.g.

chromogranin B and apolipoprotein H⁴⁴, as reproducible and clinically useful. Another major issue is that among >3,000 proteins identified in human CSF, those related to the CNS, structurally or functionally, are in minority⁴⁶. The proteins specific to PD pathogenesis are even few in numbers.

In our opinion, the slow progress to identify unique CSF biomarkers in PD resulted from two major difficulties. First, the underlying pathogenic events are too complex to be accurately reflected in a single molecule. Second, PD pathology, though it becomes more diffuse in advanced stages of disease, is still relatively localized and the tissue/disease specific markers may be so dilute in CSF, a generalized pool of brain metabolic output, that they are very difficult to find unless being looked for specifically. To mitigate this limitation, tissue markers in pathologically involved brain regions that correlate with PD and PD progression need to be identified first, which is why we believe an animal model could be helpful (Fig 1). It is apparent that not all proteins identified in tissues will be present in CSF. In addition, as PD pathogenesis is largely unknown, the brain regions examined may not contain all of the protein markers in CSF that are diagnostic for PD or that play significant roles in PD progression. Hence, an ideal approach to discover PD biomarkers is an integrated one that involves both looking for brain region-specific markers and nonbiased profiling of optimally processed CSF proteins in well-characterized PD patients as well as asymptomatic subjects as discussed above.

Metabolomics—Until recently, the most promising metabolic marker for PD was the demonstration of an inverse correlation (see earlier discussion on this topic as well) between the concentration of urate, an endogenous antioxidant⁴⁷, and clinical progression of PD, with reduced urate levels suggesting an increase in DAergic neurodegeneration and advanced PD symptomology^{34 48_51}.

With introduction of metabolomics to PD research, several groups have explored the disruption of metabolic pathways in PD by analyzing human blood for metabolic signatures. The first study by Bogdanov et al.⁵² revealed a few alterations in metabolites associated with the oxidative stress pathway, including decreased uric acid, as well as increased glutathione and 8-OHdG. A more recent study by the same group elaborated upon their previous findings and used metabolomics to evaluate the metabolic profiles of patients with sPD, PD associated with the G2019S LRRK2 mutation, asymptomatic LRRK2 G2019S carriers, and normal controls⁵³. Again, they were able to distinguish sPD cases from control, based on levels of metabolic signatures. Even more interesting, they reported a clear delineation between sPD and the LRRK2 PD group. Furthermore, a partial separation of LRRK2 PD patients and asymptomatic relatives who are carrying the LRRK2 mutation was also seen, although a substantial overlap between the two groups was present. This observation is consistent with our hypothesis that a subset of markers identified in LRRK2 patients can be applied to sPD patients.

One obvious issue (that is not necessarily unique to metabolomics) is: generally speaking, there is a lack of good reproducibility among different “-omics” investigations. While some variability is biological, e.g. the overall heterogeneity of the human population, a significant contribution to the inconsistent results relates to variables involved in experimentation with different technologies and incompleteness of current database for proteomics and metabolomics. Some of these issues can be resolved when less focus is put on the agreement of specific products and instead the entirety of the pathway within which particular genes/proteins/metabolites reside is considered. Indeed, when considering transcriptomics and proteomics, similar pathways have been shown to change between control and PD subjects, including synaptic transmission, mitochondrial function, protein degradation, and oxidative stress. Interestingly, when this approach is applied it appears that alteration to the oxidative

stress pathway is a common feature discovered in transcriptomic, proteomic, as well as metabolomic studies of PD. Moreover, while the transcriptomic and proteomic studies discovered this alteration in human midbrain tissue, metabolomics uncovered this pathway in human serum. Given the agreement of this finding through three independent means in addition to different body tissues, these data provide a strong argument for the role of oxidative stress in the pathogenesis of PD. It should be emphasized that several altered proteins, e.g. mortalin, revealed by proteomics in PD patients^{54,55} are clearly related to mitochondrial function, and therefore, are probably worth further investigation by targeted investigation.

4) Concluding remarks and future directions

Advances, whether targeted testing or unbiased profiling, have been made in PD biomarker discovery over the last few years. However, many challenges lie ahead. First, there is the lack of widely used standardized operating procedures (SOP) for sample processing⁵⁶, making it very difficult, if not impossible, to compare the results obtained by different groups. Moving forward, it is essential to process the samples as quickly as possible for any protein or metabolite studies of CSF (or any other biological fluids). It is clearly important to compare the results among different studies when the same sample protocol is followed. It has also been shown that the procedure length in CSF collection and the type of storage tube used will influence protein values, especially when analyzing lipophilic proteins (like β -amyloid and α -synuclein). This discovery was first made in CSF β -amyloid research⁵⁷. Processing time, temperature, storage conditions and pH shifts can alter outcome, chiefly through the process of proteolysis. Standardization of the assays is essential before meaningfully tackling pre-motor markers by multiple groups.

It is also important to realize that PD (AD or any other major neurodegenerative disorders for that matter) is not a single disease; there are many sub-types of diseases in PD syndrome. In other words, it is expected that subgroups of PD patients could express different markers, i.e. a panel of markers will be needed for PD diagnosis/progression. This issue becomes more important in dealing with pre-motor markers - identifying several markers with a small 'signal' on their own in the early stage of the disease could provide a substantial signal when used in combination. This approach has been used extensively in cancer research. For instance, in a proteomic study, Petricoin et al. found that combining only 5 of over 1,200 protein candidates achieved a sensitivity of 100% with specificity of 95%⁵⁸. Similarly, Blennow and associates have used tau, phosphorylated tau, and β -amyloid simultaneously in human CSF to differentiate AD from controls as well as other types of dementia⁵⁹. We have also recently used a combination of eight markers to classify PD with high sensitivity and specificity⁶⁰.

Finally, while most current clinical assays for PD (or AD) biomarkers are purely based on antibodies, other new platforms, e.g. multiplexed immuno-PCR and mass spectrometry, quickly become available to major medical centers and hospitals. Mass spectrometry techniques, e.g. multiple reaction monitoring (MRM)⁶¹, deserve special attention since they do not need specific antibodies.

In summary, by applying advanced technology and standardization of the protocols and assays, we believe pre-motor biochemical biomarkers can be discovered in populations at risk for developing PD. Using appropriate animal models can also facilitate this incredibly important process.

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Figure 1.