Supplement 1

Genetic status

Compared to Huntington's disease (HD), which represents a solely monogenic disorder due to mutations in a single gene, a genetic cause of Parkinson's disease (PD) is found in only a fraction of PD patients with clinically typical parkinsonism. Pathogenic variants in the *SNCA*, *LRRK2*, *VPS35*, *CHCHD2*, *PRKN*, *PINK1*, and *PARK7* gene or a significant predisposition due to pathogenic variants in the *GBA* gene collectively account for ~15% of all patients with clinically typical parkinsonism. While monogenic PD is not only characterized by notable genetic heterogeneity, for several genes, the likelihood of developing PD can also vary with respect to the type of variant involved in a given gene.

Indeed, pathogenic HD and PD gene variants both share the phenomenon of reduced penetrance, which has been incorporated in the recent biological definition and staging of HD[1]. Reduced penetrance is a well-documented conundrum in hereditary diseases, where it initially emerged from case and family studies of monogenic disorders[2]. Formally, 'penetrance' is defined as the conditional probability of being affected with disease X given a specific pathogenic genotype.

While in HD, a trinucleotide repeat expansion disorder, penetrance is estimated based on the repeat length[2], penetrance of pathogenic variants differs across genes and mutation types in PD and can be classified into three groups:

- Group 1) Fully penetrant variants are dominantly inherited *SNCA* triplications and missense variants, as well as recessively inherited *Parkin*, *PINK1*, and *PARK7* missense and copy number variants (CNVs).
- Group 2) Pathogenic variants with reduced penetrance but still conferring a strong predisposition to PD comprise dominantly inherited *SNCA* duplications, as well as pathogenic variants in *LRRK2* and *VPS35,* and *CHCHD2*.
- Group 3) The third group consists of heterozygous severe *GBA* pathogenic variants that are associated with an intermediate predisposition to develop PD.

Mild *GBA* pathogenic variants, risk variants, and polygenic risk scores also all increase the risk of developing PD but are not considered for the definition of a G^+ or G^- group in the present approach due to their much smaller and individually unpredictable effect size.

Conscious of the fact that monogenic conditions of PD may have a long preclinical period starting as early as birth (or even conception), the field of hereditary movement disorders is beginning to adapt its classifications to consider this phase as the earliest disease stage. For example, the recent biological classification of HD characterizes individuals for research purposes from birth starting at Stage 0, i.e. individuals with a fully penetrant pathologically expanded repeat but without any detectable pathological disease manifestation as yet[1]. In a similar vein, the present biological definition of PD considers carriers of fully penetrant pathogenic PD variants as having "Genetic PD", comparable to Stage 0 in HD, whereas Groups 2 and 3 require the presence of additional features to allow for a biological definition of PD.

To reflect this in our nomenclature, we propose a subdivision into G_F and G_P, with G_F designating the above-mentioned fully penetrant variants in the respective genes (Group 1) and G_P encompassing pathogenic variants resulting in reduced penetrance but a

considerable predisposition to developing PD that is either strong (Group 2) or intermediate (Group 3).

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Supplement 2

Synuclein status

Many biological pathways are postulated to be involved in the neurodegenerative process that underlies Parkinson's disease (PD). These include protein misfolding, aggregation, and accumulation, endosomal-lysosomal dysfunction, mitochondrial dysfunction and oxidative stress, membrane and intracellular trafficking defects, alterations in protein posttranslational modifications (PTMs), alterations in synaptic transmission, alterations in lipid and vitamin metabolism, and the imbalances in immune and inflammatory responses. It is not known to what extent dysfunction in each of these pathways contributes to individual cases of PD, nor is it known whether these changes occur in series or in parallel, or at what stage(s) in the disease process they take place and for how long. Given the well-known marked heterogeneity of PD (e.g., clinical, genetic, pathologic) it is likely that there is also a great deal of heterogeneity in the pathogenesis and it has been argued that, rather than dealing with PD as a homogenous disorder, future "precision medicine" approaches will need to identify PD subtypes based on biomarker-driven molecular phenotypes that reflect diverse cellular processes.

Numerous studies have attempted to evaluate biomarkers for PD, in many cases inspired by the Alzheimer's disease (AD) field. These include measurement of markers of neurodegeneration, neuroinflammation, protein aggregation, or proteostasis network components (Table S1). Nevertheless, until very recently, none have been proven to distinguish between PD and controls reliably and definitively, nor between PD and other neurodegenerative parkinsonian disorders, partially due to our limited understanding of the biological heterogeneity mentioned above. The advent of various 'omics' platforms, enabling the profiling of hundreds or thousands of molecules at the same time, is now also being applied in the field of PD. In this context, genomic studies aimed at identifying specific gene expression signatures (including both coding or non-coding RNA signatures) in PD are highly promising, as they may not only enable early diagnosis, but also to distinguish between different pathogenic subtypes (1), (2), (3). Importantly, these studies can now be easily performed in a variety of biological samples, such

as blood/serum, CSF, or other biofluids. Likewise, metabolomic studies are highly promising as they enable the measurement of numerous metabolites at once in the same type of biological samples. Several studies using patient-derived CSF or serum have been performed, and are starting to shed light into disease associated pathways, in addition to affording the possibility of defining metabolite signatures that may distinguish between different PD subtypes (4), (5), (6), (7). However, given the biological heterogeneity, the complexity of the technological approaches needed, the inter-laboratory variability in the approaches followed, and the need for cross-validation by different laboratories, these approaches are still not ready for prime-time, and additional studies are needed before these types of biomarkers can be recommended.

In contrast to this biological heterogeneity, the pathology of PD, with selected exceptions (see below), is defined by the presence of widespread aggregated α-synuclein (α-syn) as Lewy bodies and Lewy neurites (Lewy pathology) in the central and peripheral nervous systems. It is believed that this is an early feature of the disease, established well before clinical manifestations may become evident (8),(9),(10), (11), (12), although it is also clear that the severity of α-syn aggregation does not correlate with the severity of neuronal degeneration, nor is aggregation of α-syn required for neuronal loss (13),(9). These latter immunohistopathological findings may in part be explained by the presence of other, perhaps more toxic, forms of α-syn (e.g., oligomers) that are not evident as proteinaceous aggregates in post-mortem studies, at least using current standard imaging techniques. Whatever the explanation, it is now widely believed that the deposition and spread of pathologic forms of misfolded α-syn are key aspects in the development and progression of the neurodegenerative process and this is further supported by the established relationship between PD, α-syn pathology and common and rare variants and multiplications of the *SNCA* gene (14).

Conceptual biological definition: S^+ and S^- PD

Given the central role of α-syn in defining the pathological diagnosis of PD, as well as the overwhelming evidence that it is a strong contributor in selected genetic forms of PD and might be a key driver of neurodegeneration in sporadic PD, it is logical that *in vivo* assessment(s) of α-syn should serve as a principal component of the biological definition of PD. Therefore, we propose that pathological α-syn should be a defining attribute of the biological disease definition (i.e., an α-syn positive or negative ($S^{+/-}$) designation).

All individuals classified in a new biological definition as having sporadic PD would necessarily be S^{+} (i.e., having a positive result on an ideal 100% sensitive /specific α-syn assay). However, some genetic causes in patients currently classified clinically as PD may lack α-syn aggregation (e.g., specific autosomal recessive forms such as parkin-PD and a proportion of LRRK2-PD cases). Combining criteria for Genetic and Neurodegeneration Categories with the Synuclein status in the composite biological definition of PD accommodates the inclusion of these patients into an overarching diagnostic scheme. Thus, *α-syn-negative forms of PD (S^{-})* are included in the biological definition of PD, if genetic markers with high penetrance are present (see below for currently known high-penetrance genes).

Defining Synuclein status during life: Current state of the art

A variety of methods have been used to evaluate the presence of presumed pathogenic α-syn *in vivo* in biological fluids (CSF, saliva, blood, tears) and tissues (e.g., skin, salivary glands, gastrointestinal tract, olfactory mucosa). To date, although promising preliminary results have been reported in multiple system atrophy (MSA) (15), there is still no imaging method established for the detection of pathological α-syn in the central or peripheral nervous systems. The development of a reliable imaging ligand for α-syn is a high-priority unmet need in the field. The following sections will briefly review the various methods and samples used to assay α-syn and provide pros and cons for considering each of these in the final choice of acceptable markers of the presence of this critical component of the biological definition. Overall, for

acceptance of an assay as a marker of α-syn positivity for the purposes of biologically defining PD, although the sensitivity is obviously important, the specificity in particular needs to be consistently very high (i.e., in the range of 90% or more). Currently, these criteria are only met by seeding amplification assays of the CSF and skin and possibly by immunohistofluorescence of the skin. Emphasizing the importance of differentiating Lewy body diseases from MSA we have chosen the term "Parkinson's-type synucleinopathy" to designate S positivity based on sensitive and specific laboratory criteria for (we have not used the term PD here to avoid confusion with clinical features of the disease which are considered in the C criteria).

α-Syn level assays

Assays measuring α-syn levels in biological fluids emerged to test the hypothesis that the aggregation of □-syn in LBs and LNs might change the levels of soluble protein in these fluids, in analogy to what has been reported for A□ peptide and tau in the CSF of patients with AD. The hypothesis that □-syn pathology spreads in a prion-like manner also supported the idea that, at some point in the disease process, there should be a pool of □-syn present in the CSF, and in other biological fluids, that may reflect pathological alterations. However, it is important to keep in mind that □-syn aggregation is not specific for PD, as it accumulates in dementia with Lewy bodies (DLB), MSA, and even in diseases that are not traditionally considered synucleinopathies, including AD.

The development of various sensitive ELISA assays enabled the initial measurement of total □-syn in CSF and the verification of lower levels in PD and in other synucleinopathies (16), (17). With the emergence of more sensitive technologies, such as electrochemiluminescence-based assays, and single molecule array detection assays, it was possible to measure α-syn levels in other biofluids, such as blood/plasma, saliva, and tears. However, given the variability in the findings, differences in sample preparation and handling between laboratories needs to be carefully considered and standardized. At present the methods used for □-syn level assays lack

the ability to discriminate between different synucleinopathies which limits their value as a tool for defining PD. Additionally, the hypothesis that oligomeric species of □-syn might be more toxic than larger aggregated forms has encouraged attempts to measure the levels of □-syn oligomeric species in biological fluids. One major issue in the literature, is that the term 'oligomeric species' is still ill-defined, making it difficult to know which species are more disease-relevant and should be measured.

The identification of post-translationally modified (PTM) forms of □-syn in LBs was an important step in our understanding of possible pathological mechanisms in PD and, more generally, in synucleinopathies. In particular, phosphorylation on serine 129 (pS129) is widely used as a marker of pathology. Subsequently, the identification of other PTMs of □-syn in the brain suggested that measuring the levels of modified □-syn in various biofluids might also hold value as putative biomarkers (18). However, attempts at correlating levels of modified □-syn with disease have so far have been inconclusive.

Importantly, measurements of these PTM forms of □-syn are limited by the availability of adequate "tools", such as antibodies, that afford sufficient specificity and sensitivity, requiring the development of better tools to enable discrimination between different synucleinopathies.

In summary, our present view is that, measuring□□-syn levels in biological fluids cannot be recommended at this time as a molecular marker of a biological diagnosis of PD. We speculate that, if combined with other assays or biomarkers, these measurements may aid in the stratification of patients for future research including enrollment in clinical trials.

Immunohistochemical methods

Post-mortem studies using immunohistochemical (IHC) methods to detect phosphorylated α-syn in brain and peripheral organs have led the way to performing tissue biopsies in living

patients. In post-mortem studies of peripheral tissues, the highest IHC density of α-syn was found in the submandibular gland (SMG) as well as a rostro-caudal gradient in the gastrointestinal (GI) tract with lower density in the skin (19),(20). While initial biopsy studies concentrated on the SMG and GI tract, changes in methods have led to the skin becoming the most studied peripheral tissue biopsy site (21).

A major factor when trying to compare results from published biopsy studies has been the great variability in methods. These include differences in tissue thickness, whether the tissue is fresh or frozen, tissue preparation (fixed, paraffin embedded), the specific antibodies used to stain the tissue, distinguishing staining of nerve elements versus non-specific staining, and whether light microscopy or fluorescence microscopy was used (21),(22). Neuropathologist experience and training is also a critical documented issue (23). Very few groups have published detailed methodology papers comparing techniques and determining inter-rater reliability (24),(25). Some studies have required all raters to agree on positivity of a sample and others reassessed samples to come to a consensus. These varying methods have hampered the field. Most studies have been quite small, and while the aggregate of all studies is enough to draw some conclusions, larger studies using the same methodology (including more automated methods (e.g., (26)) are needed to ensure that diagnostic accuracy is sufficient to recommend these biopsies. The location of the α-syn is also a critical issue and most studies appear to show that α-syn is in autonomic fibers in PD but in somatic fibers and possibly in Schwann cells in MSA (27),(28). Another major limitation of biopsy studies is a lack of autopsy confirmation to affirm the diagnostic accuracy of the results.

Initial biopsy studies of the SMG in PD patients from three different groups found good sensitivity (68%) and excellent specificity ((93%) but were limited by obtaining adequate tissue from only 75% of cases (29),(30),(31). This procedure is also limited by the need to have a head and neck surgeon, or other specialized physician, perform the biopsies. Biopsies of the labial salivary gland provided adequate tissue in all cases but sensitivity was only 28% while specificity was 93% (32),(33),(29).

The presence of α-syn detected by IHC in colon biopsy samples from patients with PD, and even in samples that had been taken prior to the onset of PD motor signs, was initially very promising. Colon biopsy studies have used multiple different methods including tissue biopsy depth (not being able to safely biopsy deeper than the submucosa), antibody used, etc. accounting for quite variable reported results. In a meta-analysis of 12 studies the pooled sensitivity was 43% (37% to -48%) and the pooled specificity was 82% (78% to 0.86%) (34). Given the better results for skin (see below) most groups have abandoned studying colon biopsies for diagnostic purposes. Post-mortem studies have shown that there is a rostro-caudal gradient within the GI tract with the esophagus and stomach having the highest α-syn detected by IHC. However, one study did not find a difference in α-syn detected by IHC in gastric biopsies of 38 PD patients (31% positive) compared to 53 healthy controls (33% positive) (35).

The majority of tissue biopsy studies, especially in the past 5 years, have evaluated the skin with variable results. As with the other tissue biopsy IHC studies, multiple different methods have been applied and few have reported inter-rater reliability (36). Published studies have used skin biopsies of different depths, multiple different skin locations with some showing a rostro-caudal gradient (cervical, abdominal, arm, upper leg, distal leg), different tissue preparations (thick and free-floating vs thin mounted sections), fixed vs frozen tissue, different thickness of the cryosections, different antibodies for staining, and the use of light microscopy vs fluorescence microscopy. In a meta-analysis of 9 studies of skin biopsies from PD patients the pooled sensitivity was 76% (69% to 82%) and the pooled specificity was 100% (98% to 100%) (34). Multiple studies in patients with isolated rapid eye movement behavioral disorder (iRBD) have shown fairly high sensitivity, suggesting this can be used as a very early diagnostic marker of synucleinopathies in general (37), (38),(39).

Only one study has compared IHC for □-syn in biopsies from multiple tissues in the same patient (40). The sensitivity for SMG, skin, and colon was 56.1%, 24.1%, 14.0%, respectively, while the

specificity was 92.9%, 100%, 100%. Methods differed from studies showing higher sensitivity in the skin.

The data for IHC studies suggest that currently only skin biopsies, with specific documented methods by a limited number of groups, would provide adequate sensitivity and specificity to be used as part of the Biological Definition of PD. These biopsies are widely available, do not require a physician to perform them, provide adequate tissue for testing in almost all cases, and have minimal side effects. However, given the tremendous variability in methodology (and consequently, results) it will be critical that labs used for this testing have performed and published adequate sensitivity, specificity, and reliability data. Further, given these issues, including the time required to process and evaluate the samples, it is not clear whether this approach to diagnosis could be reliably scaled to make it a practical method of screening large numbers of samples to establish a biological diagnosis of PD.

One approach to dealing with the limitation imposed by pathologist time, effort, and training would be the development of artificial intelligence methods for screening biopsies. For example, deep machine learning and a convolutional neural network was recently used to identify α-syn in whole slide images from submandibular gland biopsies (41) and this type of methodology may aid in quantitation of α-syn, may speed processing, and may permit larger numbers of samples to be screened.

Seeding Amplification Assays

Seeding amplification assays (SAAs) are novel ultrasensitive technologies for amplifying and detecting minute amounts of misfolded proteins. Initial reports involved studies in transmissible spongiform encephalopathies using protein misfolding cyclic amplification (PMCA) to assess the presence of pathological prion proteins. Subsequent modifications, replacing sonication with vigorous shaking and combining thioflavin T fluorescence assays, resulted in the

more widespread application of the real-time quaking-induced conversion (RT-QuIC) method of seeding amplification of misfolded proteins. These methods are now established as the core diagnostic technique in sporadic Creutzfeldt-Jakob disease, capable of detecting femtogram amounts of misfolded prion proteins with high specificity (42), (43). Over the past decade substantial evidence has been amassed for the presence of selected misfolded proteins in neurodegenerative diseases and for a pathogenic role of these in inducing a prion-like inter-neuronal spread of the disease and potentially driving the neurodegenerative process. SAAs reliably detect pathogenic α-syn in post-mortem brains and peripheral tissues of patients with PD and other synucleinopathies ((DLB, incidental Lewy body disease (ILBD) and MSA). More recently, these techniques have been studied in living patients in a variety of biospecimens (CSF, olfactory mucosa, submandibular gland, gastrointestinal tract, saliva, skin, blood), in different clinical-pathological phenotypes (PD, DLB, MSA), as well as in individuals with "prodromal" forms of these diseases (iRBD
(44), (45), (23), (46) pure autonomic failure (PAF) (47), and mild cognitive impairment due to Lewy body disease (MCI-LB) (48) comparing cases to both healthy controls as well as patients with non-synuclein-related neurodegenerative diseases (e.g., AD, progressive supranuclear palsy, corticobasal degeneration and other frontotemporal dementias). Overall, the results of these studies are highly consistent and supportive of the ability of SAAs to detect *in vivo* abnormal α-syn aggregates in most patients with neurodegenerative synucleinopathies (see (49) for review).

In a very recent meta-analysis of 36 studies involving 2,722 patients with synucleinopathies (some diagnosed clinically and others pathologically) and 2,278 controls (a combination of healthy and diseased controls) Yoo et al (50) found the sensitivity and specificity of α-syn-SAA positivity for the diagnosis of α-synucleinopathies were 88% (95% CIs = 84% to91%) and 95% (93% to 97%), respectively. These were consistently higher for pathologically confirmed cases, probably due to the exclusion of incorrect diagnoses. Although it might also be argued that the latter finding relates to the possibility of greater amounts of pathological α-syn present in later stages of the disease, this is unlikely given the high sensitivity (90% (79% to95%)) and specificity

(96% (90% to 98%)) in some (but not all, e.g., (23)) studies of prodromal α-synucleinopathies (e.g., iRBD). Skin and CSF showed equivalent and consistently high sensitivities (0.92(0.87–0.95) and 0.90 (0.86–0.93) respectively), while olfactory mucosa had a significantly lower sensitivity 0.64 (0.49–0.76) compared to skin and CSF. Recent limited studies of RT-QuIC in saliva have also found somewhat lower sensitivity and specificity (51), (52). In the meta-analysis, the diagnostic sensitivity and specificity of CSF α-syn-SAAs were 0.91(0.87–0.94) and 0.96(0.93–0.98) for Lewy body disease (PD, DLB) compared to 0.63(0.24–0.90) and 0.97(0.93–0.99) for MSA (see below).

Given these results, we believe that α-syn-SAA positivity, particularly in the CSF and skin (although the other tissues/fluids mentioned above remain under active investigation), is now a sufficiently strong marker for pathological α-syn that it can be incorporated into a biological definition of PD with some caveats. The number and location of the skin biopsies increase the positivity rates. Most studies that have evaluated samples from different body regions report the highest diagnostic accuracies in posterior cervical samples (e.g., (53), (54)). Some groups report equal or slightly greater sensitivity of IHC/IHF methods in the skin (55) while others have found RT-QuIC to be more sensitive (46). This is a rapidly evolving field, and we expect that future improvements and standardization of methods are inevitable. For example, varying the type synuclein substrate or buffering conditions used may provide differing results (56). Both RT-QuIC and PMCA provide valid results, although some have argued that RT-QuIC is faster and somewhat more consistent and reliable (e.g., (53)). Obviously, accessibility issues will strongly favor the wider application of skin biopsy over CSF in the biological diagnostic approach. The main parameters of the RT-QuIC kinetic curves (e.g., lag phase, rate of change, I_{max}, and area under the curve) have been extensively evaluated with respect to synucleinopathy diagnosis or clinical correlates (e.g., motor scores) with inconsistent results. However, these parameters are greatly influenced by altering the reaction buffering conditions (using an array of pHs, ionic strengths), and applying these techniques it may be possible to optimize RT-QuIC conditions to reliably distinguish between different α-syn strains, particularly separating MSA from PD. In this regard, although most groups (e.g., (57),(58), (59)) have not been able to reliably demonstrate RT-QuIC α-syn positivity in MSA samples (accounting for the low sensitivity described above),

such methodological differences probably account for the clear distinction, based on lag phases and maximum fluorescence value at the plateau of aggregation, found in the SAA kinetic curves for MSA and PD in brain and CSF by at least 2 groups (60), (61). However, initial attempts to distinguish between PD and MSA on the basis RT-QuIC kinetics in skin samples have not been successful (62).

This raises a critical issue with respect to accepting RT-QuIC α-syn positivity as a biological criterion for the diagnosis of PD: the method(s) used (including the specifics of the sample-type evaluated) must be shown to consistently distinguish PD from MSA. One approach would be to exclusively apply methodology that is reliably negative in MSA, however, this would limit its utility in also being able to confirm a diagnosis of MSA (for clinical and research purposes). Alternatively, and we would argue preferably, would be to use a method and sample type that can reliably differentiate the positive seeding of PD from MSA. However, to date this has been reported only using PMCA in the CSF (e.g., (60)). Some studies have combined assessment of RT-QuIC α-syn with neurofilament light chain (NfL) levels in the CSF (58), (59) to differentiate MSA from PD. Given the practical advantages of obtaining skin biopsies over CSF, one study that applied an RT-QuIC method that is positive in the skin in both PD and MSA (62), combined this with serum NfL levels to distinguished PD (normal NFl levels) from MSA (elevated NfL levels). One could also consider adding imaging or other exclusionary criteria supportive of MSA (63) in this diagnostic process. Another possible approach would be to combine SAA with IHC techniques. As discussed above, methods of evaluating α-syn immunoreactivity in the skin have demonstrated different patterns of distribution (autonomic neurons in PD and somatic neurons and Schwann cells in MSA). However, the limitations of methodological variability and scalability mentioned above may limit this approach.

It is expected that eventually SAA and other technologies will permit the development of reliable blood-based biomarkers for PD, precluding the need for lumbar puncture and even skin biopsy. For example, a recent SAA study has demonstrated pathological α-syn in neuronal derived exosomes from the blood of patients with PD (64). It is also possible that studies of the

structure or seeding characteristics of the aggregates amplified by the SAAs may enhance diagnostic reliability (e.g., differentiating the different synucleinopathies using cryo-EM (65),(66) and possibly provide insights into Lewy body disease clinical heterogeneity (67) and possibly even allow the development of more targeted personalized therapies. It is also expected that advances in these and other methodologies will move beyond a binary +/- diagnostic test to techniques that could be used to monitor disease status and progression. For example, a recent study combining an oligomers-specific ELISA quantification of the CSF α-syn SAA end product found a significant correlation with clinical severity measures (68). As these techniques advance it is expected that SAAs will be incorporated into clinical trials in a number of ways, not only as an initial confirmation of diagnosis, but also defining the presence of co-pathologies both for trial exclusion as well as inclusion depending on the mechanisms of action of the therapeutic agent(s), in monitoring disease status, and assessing target engagement (69).

Table S1. Non-Synuclein-based fluid biomarkers studied in PD and in other parkinsonisms.

Measurement	Biological Relevance	Biological Fluid(s)	Outcome	References
Neurofilament light (NfL) and phosphorylated NfL (pNfL)	Marker of neurodegeneration	CSF, serum	Increased in patients with ND vs. HC; increased in MSA vs. PD and DLB; Cannot reliably discriminate between parkinsonisms.	(70) (17)
Glial fibrillary acidic protein (GFAP)	Marker of neuroinflammation	CSF	Increased in ND vs. HC. Non-significant in discriminating between parkinsonisms.	(70) (17)
Soluble TREM2 receptor (sTREM2)	Marker of neuroinflammation	CSF, serum	Increased in ND vs. HC. Non-significant in discriminating between parkinsonisms.	(70) (17)
Interleukin-6 (IL-6)	Marker of neuroinflammation	CSF	Non-significant between PD and HC.	(70)
Chitinase-3-like protein 1 (YKL40)	Marker of neuroinflammation	CSF, serum	Increased in AD. Non-significant in discriminating between parkinsonisms.	(70) (17)
S100 calcium binding protein B (S100B)	Marker of neuroinflammation and regulation of various cellular processes	CSF, serum	Increased in ND vs. HC. Non-significant in discriminating between parkinsonisms.	(70) (17)
Lysosomal enzymes	Marker of autophagic activity	CSF, blood	Increased in ND vs. HC. Non-significant in discriminating between parkinsonisms.	(71)

Aβ peptides	Biomarker in AD	CSF	Decreased in AD vs. HC. Non-significant in discriminating between parkinsonisms.	(17)
Tau	Biomarker in AD	CSF	Increased in AD vs. HC. Non-significant in discriminating between parkinsonisms.	(17)

PD, Parkinson's disease; AD, Alzheimer's disease; CSF, cerebrospinal fluid, ND, neurodegenerative disease; HC, healthy control;

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Supplement 3

Neurodegeneration status

Conceptual definition of N^+

Neurodegeneration in PD (N+) is conceptualised to occur after initiation of causal biological mechanisms and may be present before onset of clinical signs and symptoms. Pathologically it is characterised by neuronal loss and gliosis which are variably associated with microglial activation and other indices of neuroinflammation [1,2]. Currently, neuroimaging techniques are the only approaches that can provide direct evidence for neurodegenerative changes in PD, involving the nigro-striatal dopamine system as well as in other brain areas or the peripheral autonomic nervous system [3]. Therefore, we recommend to define the N+ state by imaging procedures, although in the future, non-imaging methods like tissue biopsies or molecular neurodegeneration markers may evolve.

Current approaches and limitations

Molecular imaging (PET; SPECT)

Dopaminergic Imaging
Molecular imaging of the presynaptic dopamine system using a variety of PET or SPECT radioligands for the dopamine transporter (DAT), L-aromatic acid decarboxylase (i.e. 6-^{18}F-fluoro-L-dopa, F-dopa) or the vesicular monoamine transporter type 2 (VMAT2; ^{11}C- or ^{18}F-labeled dihydrotetrabenazine) are all sensitive to detect a reduction in nigro-striatal dopamine nerve terminals, and to a more variable degree, loss of signal in the substantia nigra. However, while a positive scan can generally differentiate between degenerative parkinsonism and health or other potential mimics such as essential tremor or neuroleptic-induced parkinsonism, the specificity for the most challenging clinical situation (i.e. differentiating between PD and atypical parkinsonism such as MSA or PSP) is poor. Furthermore, a negative scan may not be fully reassuring, as subjects who have otherwise unexplained clinical parkinsonism and a normal scan may have other non-motor features of PD [4] that may progress over time [5] and may also show clinically important change in motor function [6]. Furthermore, the relationship between DAT SPECT and dopaminergic nerve terminals and cell counts is unclear [7,8]. In the case of F-dopa, this may on occasion reflect tracer uptake by remaining serotonergic neurons [9].

Non-Dopaminergic Imaging
Several non-dopaminergic tracers have been used in PET studies of clinically established PD and to a lesser extent also in subjects with iRBD as an example of early disease stage [3]. *Serotonin transporter ligands* like 11C-DASB bind to presynaptic terminals and are sensitive to serotonergic degeneration [10]. Reduced binding has been reported both in early PD and in pre-symptomatic carriers of SNCA mutations[11], while upregulated binding has been observed in asymptomaric carriers of LRRK2 mutations [12]. *Cholinergic denervation* has been demonstrated in early PD and also at the level of the gastrointestinal tract in early PD using PET ligands of acetylcholine esterase[13].

Cardiac imaging with either [^{123}I]MIBG SPECT or [^{18}F]dopamine PET can detect loss of post-ganglionic sympathetic innervation and may be present during early disease (i.e. RBD) or early stages of PD [14]. As the sympathetic defect in MSA is predominantly pre-ganglionic, cardiac imaging may also help differentiate between PD with autonomic dysfunction and MSA. However, up to 44% of MSA patients may have abnormal MIBG binding[15-18] and conversely up to 61% of early stage PD patients have normal MIBG SPECT results[18].
In addition, sympathetic cardiac imaging can be confounded by a variety of factors including cardiomyopathy, cardiac denervation unrelated to parkinsonism, or effects of medication interfering with tracer binding[19].

Metabolic imaging using FDG PET, while *chemically* non-specific, is able to reveal PD-specific functional changes that are sensitive to early disease and effective at differentiating amongst different forms of degenerative parkinsonism [20,21], although it is unclear how tightly they are coupled to neurodegeneration and one recent paper has suggested that similar changes may be seen in association with neuroleptic use[22].

Neuroinflammation is another molecular imaging target of potential interest in PD, but even if a robust inflammatory response is detected, it does not necessarily reflect neurodegeneration, nor would it be specific for PD [23]. Furthermore, most current tracers are based on the translocator protein (TSPO), for which first generation ligands (^{11}C-PK 11195) have poor signal to noise ratio, while the binding of the newer tracers is affected by the A147T polymorphism [24] and findings in PD are very inconsistent [25]. Glial imaging with the TSPO ligand ^{11}C-PBR28 may be useful, however, in distinguishing MSA from PD as suggested by a recent study showing characteristic patterns of increased binding in patients with MSA yielding close to 100% sensitivity and specificity [23].

Similar to Alzheimer disease, where PET imaging with tracers for Amyloid beta and tau deposition is used as an anchor for a biological definition of disease [26] a validated *α-synuclein PET* tracer that can specifically label pathological α-synuclein aggregates could potentially provide high sensitivity and specificity to detect the core pathology driving neurodegeneration in PD. Although some progress has been made in this respect , labeling α-synuclein *in vivo* is associated with numerous challenges [27,28]. To date published reports are based on only very small numbers of PD and MSA subjects and suggest potential of 2 different tracers to identify pathological α-synuclein deposition in the putamen and cerebellum of patients with MSA but limited sensitivity in PD (9; Smith R et al, abstract presented at ADPD 2022).

MR imaging

Structural MRI was traditionally regarded as relatively unrevealing in PD, but newer techniques, including neuromelanin (NM) and iron sensitive sequences as well as diffusion imaging all show changes in PD that most likely reflect neurodegeneration.

MR morphometry

Different techniques of volumetric analysis of T1-based structural MRI data have revealed areas of volume loss in cortical and subcortical areas in early PD that likely reflect neurodegeneration [29]. Areas of reduced cortical thickness and volume loss in the

hippocampus as well as subcortical nuclei like the thalamus and putamen have also been described in subjects with idiopathic RBD suggesting sensitivity to change in prodromal PD as well [30]. While recent studies using automated volumetry techniques have revealed specific patterns of volume loss in MSA and PSP enabling differential diagnosis from PD, with high sensitivity and specificity [31] the specificity of cortical and subcortical volume changes for PD appears to be low.

Neuromelanin Imaging

Neuromelanin (NM) is a characteristic constituent of dopaminergic neurons in the substantia nigra (SN) as well as of noradrenergic neurons of the locus coeruleus (LC) ands acts as a chelator of metals like iron and copper resulting in paramagnetic properties and T1-shortening effects on MR imaging [32]. High-field MR fast spin-echo sequences show NM containg nuclei as high signal intensity areas relative to the surrounding brain tissue [33]. MR imaging with NM sensitive sequences have consistently shown signal reductions in the area of the substantia nigra and also the LC and measures of signal intensity and area of high signal have yielded sensitivity and specificity for PD vs healthy controls of around 80% [34]. NM imaging probably provides the most direct evidence of loss of neuromelanin-containing monoaminergic (DA and noradrenergic) neurons. NM signal correlates with dopaminergic markers in established PD [35] and striatal dopamine release [36] and changes can be seen in the SN (and LC) in prodromal disease [37]. However, NM imaging alone may not be sufficient as a sole marker of neurodegeneration in early PD [38], nor specific for the differentiation of PD vs. MSA, although the distribution of signal changes within the SN may be different in PSP [39]. In keeping with the hypothesis that disease begins as a dying back process at nigrostriatal nerve terminals rather than in nigral cell bodies [40,41], changes in striatal DAT binding precede increased nigral iron and loss of nigral NM signal [42]. However, loss of NM signal in the locus coeruleus has been described in iRBD subjects with no evidence of parkinsonism, in whom DAT binding may still be normal [43].

Iron-sensitive MR

Neuropathological studies have consistently found increased iron content of the SN in PD and increased nigral iron is considered a biomarker of neurodegeneration in PD [44]. Conventional T2-weighted MR imaging shows the area of the SN diffusely hypointense due to its iron content and increased contrast in PD vesus controls [45]. MR imaging based approaches to quantify regional brain iron content include T2*-weighted imaging and R2* mapping and more recently quantitiative susceptibility mapping (QSM) [46-48]. Overall, studies of QSM of the SN to distinguish PD from healthy controls was found to have sensitivities and specificities of close to 90% while this was lower for R2* mapping [46-48].

There is only limited evidence for abnormal iron signal in premotor disease [37,49] or of a relationship between nigral R2* and clinical disease severity [50](but see [51]), and a recent study has suggested that abnormal nigral iron signal may not be seen in *de novo* disease, but only until dopaminergic therapy has been initiated (i.e. by which time approximately 50% of nigral neurons have been lost) and plateaus with disease progression [52].

Iron-sensitive high-field MRI contrasts have also revealed a dorsolateral ovoid area of relative signal hyperintensity against the background of the hypointense SN in healthy subjects, which has been suggested to anatomically correspond to the region of the calbindin-poor subregion of nigrosome-1 of the SNc [53]. This latter SN subregion has been

postulated to be the earliest and most severely affected by nigral cell loss in PD [54] and MRI studies have consistently reported unilateral or bilateral loss of this dorsolateral nigral hyperintensity (DNH) in PD subjects which sensitivities and specificities for distinguishing PD from healthy controls of above 90% [55,56]. In addition, loss of DNH has also been reported in 40 to 70% of patients with RBD , suggesting possible sensitivity to nigral degeneration during prodromal stage of disease [57]. Limitations of this MR imaging sign as a marker for neurodegeneration in PD include lack of objective, observer-independent assessment, susceptibility to artefacts and lack of specifity for PD as compared to other forms of degenerative parkinsonism.

Diffusion imaging

Diffusion imaging is sensitive to changes in the degree and directionality of intra- and extra-neuronal water. Increased nigral free water and reduced fractional anisotropy (FA) indicating reduction of diffusion along fibre tracts compatible with neurodegeneration are seen in early and prodromal (iRBD) PD and increase with disease duration [58]. However, while increased free water in the posterior substantia nigra can differentiate PD subjects from healthy controls this is also true for other forms for degenerative parkinsonism like MSA and PSP [59]. Including extra-nigral regions (striatum, midbrain, cerebellum) in the analysis allows for automated differentiation of PD from atypical parkinsonism, and between PSP and MSA [60,61].

Multimodal MRI

Given the multiple abnormalities found in the SN and other brain regions in PD using different MR contrast and analyses it may be possible to enhance senstitivity and specificty of MR imaging data to detect early neurodegeneration in PD by the use of multimodal MR protocols combined with multivariate analysis. Currently the number of studies using this approach is still limited. One study found the best accuracy to distinguish between PD and healthy controls based on multimodal MR data to be 78% [62]. Another recent study found that QSM and posterior substantia nigra free water imaging had similar power to detect prodromal (RBD) and clinical PD, while combining the two methods increased discriminative power [63].

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Supplement 4

Biological Designations - critical appraisal

G	S	N	**Biological Designation**	Comments / Caveats
Sporadic Parkinson's Disease				
G^-	S^+	N^+	**Sporadic PD***	These will be the commonest designations. Caveat related to all C^+ states: $G^-/S^+/N_P{}^+$ but $N_C{}^-$ should not be associated with clinical parkinsonism (i.e., clinical parkinsonism would require CNS evidence of SNc degeneration while MIBG scanning may be abnormal in the absence of parkinsonism). N_P = PNS marker (i.e., MIBG); N_C = CNS neurodegeneration markers
G^-	S^+	N^-	**Sporadic Parkinson's-type synucleinopathy**	
G^-	S^-	N^+	**Non-PD neurodegeneration**	This may signify an alternative neurodegeneration, however, some individuals fulfilling all clinical criteria for PD will have negative S evaluations. Prospective studies will be required in these individuals.
Genetic Parkinson's Disease**				
$G_F{}^+$	S^-	N^-	**Genetic PD***	Fully penetrant pathogenic variants
$G_{SNCA}{}^+$	S^+	N^+	**Genetic PD***	S positivity is expected in N^+ individuals. If S^- further studies required. Caveat related to N designation: Clinical parkinsonism would require CNS evidence of SNc degeneration while MIBG scanning may be abnormal in the absence of parkinsonism.
$G_{PRKN}{}^+$	S^- or S^+	N^+	**Genetic PD***	S^- is expected in the majority of cases (possibly 80% or more). Prospective studies are required to understand the differences between the S^+ or S^- groups. Caveats related to N designation and C characteristics: Clinical parkinsonism would require CNS evidence of SNc degeneration. MIBG SPECT positivity would not be expected and several of the non-motor features are typically not seen in these patients (RBD, NOH, other autonomic symptoms, dementia).

G_{PINK1} or $_{DJ-1}+$	S^- or S^+	N^+	**Genetic PD***	Very similar to comments for G_{PRKN}^+ although the relative frequencies of S^+ or S^- is currently unknown.
G_{LRRK2} or $_{GBA}^+$	S^-	N^-	**Genetic predisposition for PD**	Pathogenic variants with strong or intermediate predisposition. The presence of clinical features (i.e., C^+) changes the biological designation to Genetic PD.
G_{LRRK2}^+	S^+	N^-	**Genetic Parkinson's-type synucleinopathy**	S^+ or S^- designations are both possible with *LRRK2* mutations (S^+ will be more common and required for "Genetic Parkinson's-type synucleinopathy" designation). Prospective studies are required to understand the differences between S^+ / S^- groups. To be designated „Genetic synuclein negative PD" currently requires N^+. Caveat related to N designation: Clinical parkinsonism would require CNS evidence of SNc degeneration, while MIBG SPECT may be abnormal in the absence of parkinsonism.
G_{LRRk2}^+	S^-	N^+	**Genetic synuclein-negative PD***	See above. The presence of clinical features (i.e., C^+) changes the biological designation to "Genetic PD".
G_{GBA}^+	S^+	N^-	**Genetic Parkinson's-type synucleinopathy**	If only CNS markers of Neurodegeneration are available (i.e., MIBG SPECT is not performed and N testing related to nigrostriatal dopamine deficiency is negative) and the patient demonstrates the presence of C_{Prob} features other than Parkinsonism (see Table 5) the biological designation becomes Genetic PD.
G_{GBA}^+	S^+	N^+	**Genetic PD***	S positivity is expected in N^+ individuals. If S^- further studies required.

* Clinical features (C_{Poss} or $C_{Prob;}$ see Table 5 for details) may or may not be present.

** See Table 1 for details on genes classified as G_F (fully penetrant: *SNCA* triplications and single nucleotide variants, *PRKN*, *PINK-1*, *DJ1*) and G_P (strong predisposition: *SNCA* duplications, *LRRK2*, *VPS35*, *CHCHD2* pathogenic variants, *GBA* severely pathogenic variants).

Abbreviations: CNS: central nervous system, MIBG: meta-iodobenzylguanidine, NOH: neurogenic orthostatic hypotension, PD: Parkinson's disease, PNS: peripheral nervous system, RBD: REM sleep behavioral disorder, SNc: substantia nigra pars compacta, SPECT: single-photon emission computerized tomography.

Supplement 5

Clinical Status

As stated in the main manuscript, in a person who has shown evidence of biologic PD (G^+ or S^+ or N^+), one next determines if clinical symptoms or signs are present (i.e. C^+). Further, if potential symptoms/signs are detected, the task becomes to determine whether these are related to the underlying biologic process of PD or are related to other causes.

A key resource in the consideration of how to operationalize our criteria was the MDS prodromal PD criteria[1,2] as they already contain a template for the interpretation of positive clinical signs of PD. The MDS prodromal criteria were created to define a specific probability of prodromal PD from the general population, with the 'probable' threshold set at 80%. They use a statistical method (the Bayesian naive classifier) to estimate the likelihood that a given individual has prodromal PD. The original MDS prodromal criteria involve three steps.

1. The prior probability of having prodromal PD is estimated based upon age.
2. Diagnostic information is obtained on as many variables as possible, including environmental risk variables, genetic risk variables, prodromal symptoms and signs, and biomarker testing. Each variable is considered as a diagnostic test, the strength of which is summarized in a likelihood ratio (LR); positive tests have a LR >1, and negative tests have a LR <1. If information is unavailable, borderline, or uncertain, the LR is not applied for that test (LR=1).
3. Once all information is collected, all LRs are multiplied by each other. The total LR is then compared to the threshold that gives a >80% probability of having prodromal PD. If met, probable prodromal PD is diagnosed.

The most recent update of the prodromal criteria included risk factors, prodromal signs, and results of diagnostic testing. The prodromal symptoms/signs were as follows:

Prodromal Markers	LR+
Polysomnogram-proven RBD or Positive RBD screen questionnaire with >80% specificity	130 2.8
Subthreshold parkinsonism (UPDRS) or Abnormal Quantitative Motor Testing	9.8 3.5
Olfactory loss	6.4
Constipation	2.5
Excessive Daytime Somnolence	2.7
Confirmed Neurogenic OH Or Symptomatic Hypotension	18.5 3.2
Severe Erectile Dysfunction	3.4
Urinary dysfunction	2.0
Depression (+/- Anxiety)	1.8
Global Cognitive Deficit	1.8

Table S5– Positive likelihood ratios in the MDS prodromal criteria revision[2]

Although the original prodromal criteria used age and risk factors to define the pre-test probability, in our current definition, the C+ state would be evaluated when there is already evidence of biological disease (i.e., G^+ or S^+ or N^+). This implies a much higher pre-test probability. The stronger the evidence of the presence of underlying biological disease the higher the pre-test probability. To define C+ in already-defined evidence for biologic PD, one could, for example, posit using a 'neutral' prior likelihood (i.e. probability = 50%). Moving from this neutral equipoise position to an 80% probability would require a total LR of 4.0. This probability would be even greater in individuals who are N+ in addition to G+ or S+).

These factors were considered in the generation of the suggested criteria for C^+. Of note, the C^+ includes all disease states (not just "prodromal" disease). Therefore, the clinical states of dementia and parkinsonism[3] were considered as identifiers of the C^+ state. In addition, clinical diagnoses highly specific for synucleinopathy, namely RBD and laboratory-defined pure autonomic failure also identify the C^+ state. These features would support a Clinical designation as "Probably related to PD" (i.e., C_{Prob}^+). Isolated other important features that are part of the MDS prodromal criteria would be considered supportive of a "Possibly related to PD" (i.e., C_{Poss}^+). Classification (Table 5 in the manuscript).

Since the remaining features are less specific, it was determined that they must be present in combination; therefore, at least two markers (in the list of "possibly related" items) must be present. To further increase specificity, markers must also have no likely alternate explanation; that is, according to the clinical impression of an expert evaluator there is no alternate condition or circumstance that is a more likely explanation for the finding than PD. There are many potential circumstances like these; for example, in an individual with a stable global cognitive deficit after a head trauma, or with lifelong anxiety/depression that was not increasing over time (i.e. a possible risk factor but not a prodromal marker), or with stable erectile dysfunction that occurred immediately after prostate cancer resection, the clinician would simply not apply that specific criterion. Note that the LR from the prodromal criteria were estimated from studies that generally did not require this clinical evaluation for alternate explanations; therefore, the true LR+ with this additional step would likely be higher than the estimates from the MDS criteria. To prevent non-specificity from combining highly-correlated markers (e.g. urinary dysfunction and erectile dysfunction might both be due to the same underlying non-PD cause), we specified that symptoms must cross multiple domains (e.g. one autonomic and one sleep symptom, one motor and one sensory symptom, etc.). Finally, when combining markers for classifying C^+ as Probably related to PD, it would be particularly important for the evaluator to consider and exclude the presence of one symptom in a defining category that could be caused by a feature in another category (e.g., erectile dysfunction or excessive daytime somnolence in a patient with new severe depression).

Although this system has the disadvantage of lower precision in probability estimates (i.e. different combinations will have different total LR and therefore different probability estimates), it was felt that this current approach would be simpler to understand and apply.

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