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REVIEW

NEWS AND VIEWS ON CLINICAL NEUROIMAGING

Clinical utility and research frontiers of neuroimaging in movement disorders

Flavio NOBILI [1] *, Eric WESTMAN [2], Rosalie V. KOGAN [3], Joana B. PEREIRA [2], Federico MASSA [1], Matteo GRAZZINI [1], Sanne K. MELES [4], Klaus L. LEENDERS [3, 4]

[1]Deparment of Neuroscience (DINOGMI), IRCCS Polyclinic San Martino-IST, University of Genoa, Genoa, Italy; [2]Division of Clinical Geriatrics, Neurobiology, Care Sciences and Society, Karolinska Institute, Huddinge, Sweden; [3]Department of Nuclear Medicine and Molecular Imaging, University Medical Center Groningen (UMCG), Groningen, The Netherlands; [4]Department of Neurology, University Medical Center Groningen (UMCG), Groningen, The Netherlands

*Corresponding author: Flavio Nobili, Department of Neuroscience (DINOGMI), Clinical Neurology, Polyclinic San Martino-IST, University of Genoa, Genoa, Italy. E-mail: flaviomariano.nobili@hsanmartino.it

ABSTRACT

Neuroimaging in Parkinson's disease (PD) and other primary Parkinsonian disorders has been increasingly used in the routine clinical work in the last years. The paradigm has changed from an "exclusionary" use, *i.e.*, to rule out causes of secondary Parkinsonism, to an "inclusionary" one, *i.e.*, finding image and network characteristics allowing to identify a specific disease. This is allowed by analyses spanning from the commonly used visual analysis to the most sophisticated postprocessing leading to the identification of covariance patterns both in morphological and functional neuroimaging. However, paralleling the advancement in covariance and connectivity analyses, the issues of standardization and harmonization of data acquisition, and image reconstruction and postprocessing among centers are emerging in the scientific community. Also, the building of scientific evidence still suffers from the lack of large, formal studies and relies on relatively small cohort studies from one or few centers. Joint actions to face these issues are now ongoing in Europe, supported by specific programs, such as the Joint Programming on Neurodegenerative Diseases (JPND). In the present review, some of the most recent and relevant achievements in the field of diffusion tensor magnetic resonance imaging (MRI), functional MRI, fludeoxyglucose-positron-emission tomography, dopamine transporter single-photon emission computed tomography and non-dopaminergic imaging in PD and primary Parkinsonisms are reported.

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Parkinson's disease (PD) is associated with the accumulation of abnormal α-synuclein aggregates in the brain into the so-called Lewy bodies. These abnormalities are thought to appear first in structures of the brainstem and olfactory system, then spread to the substantia nigra and reach neocortical regions of the temporal, parietal and frontal lobes.[1] This spreading of Lewy bodies correlates with disease progression, with the gradual accumulation of pathology starting much before a clinical diagnosis can be made.[2] In the most severe stages of the disease, most PD patients develop dementia, and Lewy bodies can be found in the entire neocortex, affecting the motor and sensory areas of the brain.[1, 2]

In the past few years, a substantial effort has been made to understand the mechanisms underlying this pattern of pathological changes in PD. Specifically, there is mounting evidence suggesting that α-synuclein deposition may lead to abnormalities in distributed brain networks.[3] This could explain the variety of motor and non-motor symptoms observed in PD, including bradykinesia, mood disorders and cognitive impairment.[4] According to Brundin *et al.*, Lewy bodies might induce

misfolding of normally folded proteins, spreading from pre-synaptic to post-synaptic neurons through a prion-like mechanism.[5] Evidence supporting this mechanism of transynaptic spread has come from patients who received a transplant of dopaminergic stem cells in an attempt to restore dopaminergic neurotransmission.[6, 7] Their *post-mortem* tissue revealed Lewy bodies within the transplanted dopaminergic neurons that shared classic features with those in the substantia nigra of the host, including α-synuclein and ubiquitin immunoreactivity.[8] Hence, PD might be associated with a progressive network disruption due to the spread of Lewy bodies across the brain through the synapses or connections.

Magnetic resonance imaging

Several studies using magnetic resonance imaging (MRI) have provided support to this view of PD as dysconnectivity syndrome. In fact, there is increasing evidence showing that structural and functional connectivity can be used as reliable biomarkers to characterize disease progression in PD.[9] Moreover, connectivity measures have also shown great utility in discriminating PD from atypical parkinsonian disorders such as progressive supranuclear palsy and multiple system atrophy.[10] As the interest in brain connectivity and network topology continues to increase in the scientific community, some of the most intriguing aspects related to the pathophysiology of PD are now closer to being understood.

Two of the most common MRI techniques used to assess structural and functional connectivity in PD are diffusion tensor imaging (DTI) and functional magnetic resonance imaging (fMRI) at the resting-state. By measuring the displacement of water molecules in brain tissue, DTI represents a powerful tool to assess the integrity of white matter fibers.[11] These molecules tend to move faster along the axons than perpendicular to them, allowing to reconstruct the white matter tracts throughout the brain. The organization of the axons and their properties determine the motion of water molecules through white matter fibers and can be characterized by different DTI measures.

MRI: diffusion tensor imaging

For instance, the fractional anisotropy (FA) is a measure that quantifies the degree of diffusivity in different directions, providing information on the organization of the tensors. This measure becomes abnormal in the presence of fiber coherence loss, demyelination or axonal degeneration.[12] In addition to FA, the overall movement of water molecules within the brain can also be measured in DTI as the mean diffusivity (MD), which becomes abnormal in the presence of changes in neuronal density or tissue loss.[13] A healthy brain is characterized by high FA and low MD in the white matter, whereas a brain of a patient with white matter pathology is normally characterized by a decrease in FA and increase in MD. In addition to FA and MD, DTI also allows calculating the motion of water molecules along or perpendicular to the main fiber direction, also known as the axial diffusivity and radial diffusivity,[14] respectively. The axial diffusivity increases in the presence of axonal damage, whereas RD increases as a consequence of demyelination.[14] Although DTI is normally used to assess abnormalities in the white matter, some of its measures, such as MD, may also be used to quantify the disruption of barriers to diffusion in the gray matter tissue,[15] providing information on tissue loss. There are different methods that make use of some or all of these DTI measures, which include tract-based spatial statistics and tractography. These methods have been applied to quantify white matter abnormalities in patients at early and advanced stages of PD and in association with motor and cognitive symptoms. For instance, using high-resolution DTI, previous studies have found strong FA decreases in the substantia nigra in patients at early disease stages.[16, 17] This is in line with consistent evidence showing that the loss of dopaminergic neurons in this region coincides with the appearance of motor symptoms and clinical diagnosis of PD.[1] Remarkably, in one of these studies, the FA values from the caudal part of the substantia nigra allowed discriminating PD patients from healthy controls with 100% specificity and sensitivity.[17] This suggests that high-resolution DTI in the substantia nigra has the potential to serve as a non-invasive early biomarker for PD. At moderate stages of PD, reductions of FA in frontal areas,[18] increases of MD in the corpus callosum,[19] and changes in FA and MD in the thalamus [15] can be found in PD patients. Finally, in more severe disease stages which are characterized not only by the presence of motor symptoms but also mild cognitive impairment (MCI) or dementia, white matter changes become widespread,[19, 20] affecting both the

anterior and posterior white matter tracts, which show prominent FA losses and MD increases. These findings are in line with the extensive gray matter atrophy normally found in parietal, posterior temporal and occipital areas in PD patients with MCI [21] or dementia.[22] Overall, the abnormalities in DTI measures in PD have been found to be clinically meaningful, being associated with a complex range of symptoms. For instance, damage in the superior longitudinal fasciculus has been shown to contribute to depression [23] and bradykinesia,[24] whereas damage in the corpus callosum has been found to have a negative impact in complex behaviors such as cognitive functioning.[25] Hence, clinical manifestations are related to diffusivity abnormalities and can potentially be used to predict prognosis and assess disease progression in PD.

MRI: functional MRI

In contrast to DTI, resting-state fMRI measures the spontaneous low-frequency fluctuations of the blood oxygen level-dependent (BOLD) signals. This technique assesses functional connectivity as temporal BOLD correlations between brain regions and can be analyzed using different approaches, including seed-based, data-driven or effective connectivity analyses. In seed-based analyses, functional connectivity is assessed between a region of interest and other brain areas or all voxels in the brain. These analyses indicate the functional coupling in the mean BOLD time course between a seed selected based on prior hypotheses and the time courses of other brain voxels.[26] Studies using this approach have found functional connectivity abnormalities between the striatum,[27] subthalamic nucleus,[28] sensorimotor cortex [29] and several brain areas in PD. Some of these changes have been associated with motor impairment [27] or the presence of specific non-motor symptoms such as visual hallucinations.[30] The most common data-driven approach to assess functional connectivity in PD is based on independent component analyses. This method separates the BOLD signal into non-overlapping spatial and time components, identifying resting-state networks that are highly consistent across subjects.[31] Most studies carried out in PD have assessed the default-mode network, which mainly comprises the precuneus, medial frontal and lateral parietal regions. Decreases of functional connectivity between the areas of this network have been found in patients with PD,[32, 33] which correlated with memory and visuospatial functions [32] and were more severe in PD patients with MCI.[33] Other networks show abnormal functional connectivity in PD-MCI and include the dorsal attention and fronto-parietal networks, which could underlie the different types of cognitive impairment present in PD.[34] Finally, there is strong evidence indicating that dopamine replacement therapy has a strong impact on functional connectivity in PD, with several studies showing that dopamine normalizes aberrant functional patterns within resting-state networks and leads to an improvement of PD symptoms.[35] Effective connectivity is another method used in the analysis of fMRI data, which quantifies the influence of one region over another and the directionality of the information flow between these regions. Examples of effective connectivity methods include dynamic caudal modeling and Granger causality analysis. In a previous study using dynamic caudal modeling the effects of deep brain stimulation in the subthalamic nucleus were assessed on the motor network in PD.[36] In that study, deep brain stimulation increased connectivity strength in the cortico-striatal, thalamo-cortical, direct and indirect basal ganglia pathways. In addition, basal ganglia afferents to the subthalamic nucleus predicted clinical status and therapeutic response to deep brain stimulation, providing a means to assess treatment outcome in PD patients.

With growing evidence showing that structural and functional connectivity reflect fundamental aspects of the brain both in health and disease, the organization of the connectome has now become the focus of recent neuroimaging studies.[37] In particular, the application of graph theory to assess the organization of the large-scale brain network has provided a crucial insight into how regions communicate with each other and how this communication becomes disrupted in neurodegenerative diseases. In graph theory analyses, the brain is represented as a graph, where the nodes correspond to brain regions and the edges represent the connections between them. Several studies using graph theory have shown that the healthy human brain is organized into a small-world topology consisting of an optimal balance between local and global connectivity.[37] Measures that reflect the local communication between brain regions include the clustering coefficient, whereas global communication can be measured using the characteristic

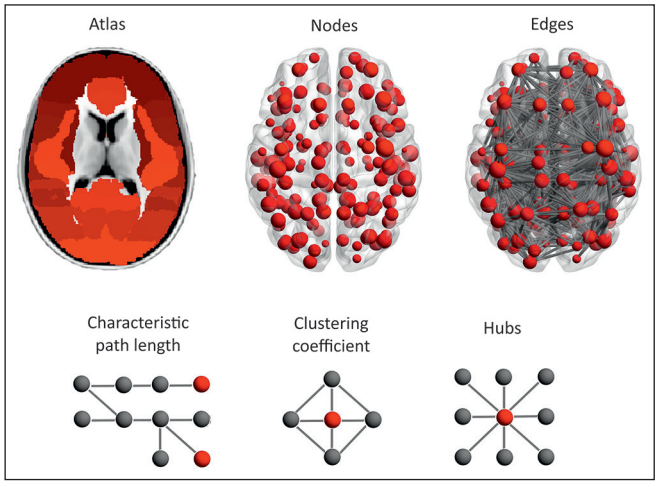

Figure 1.—Graph theory analyses are carried out by extracting a measure of interest from all regions of a brain atlas, representing them as nodes and establishing the connections between them as edges. Some of the graph theory measures found to be compromised in PD include the characteristic path length, the clustering coefficient and the hubs.

path length or global efficiency (Figure 1). [38] The characteristic path length can be calculated as the average shortest path length between all pairs of nodes in the network, whereas the global efficiency is the average inverse shortest path length. In contrast to the characteristic path length, the global efficiency may be meaningfully computed on disconnected networks, as paths between disconnected nodes are defined to have infinite length and zero efficiency.[38] The clustering coefficient quantifies the number of connections between the direct neighbors of a node as a proportion of the maximum number of possible connections. This measure reflects how well the nodes are connected to nearby regions forming clusters.[38] Studies carried out in PD have shown that these network properties are abnormal in these patients, when compared to normal controls. The characteristic path length seems to be increased in PD [39] or in patients with both PD and MCI,[40] suggesting that their networks are less integrated and characterized by longer paths or a less efficient communication between distant brain regions. The clustering coefficient has produced somewhat mixed findings; with some studies showing it is decreased,[41] increased [42] or mostly unchanged [40] in PD patients. Moreover, there is consistent evidence showing that a few brain regions play a crucial role in network communication, regulating most of the information flow by displaying a large number of connections.[38] These regions are known as the network hubs and are abnormal across a wide range of neurodegenerative disorders, including PD. In a previous study it was shown that brain regions involved in dopaminergic pathways such as the orbitofrontal cortex presented decreased hubness in PD patients,[39] being relevant to the pathophysiology of the disease. In another study, the number of hubs was dramatically decreased in PD patients, particularly in those with MCI, with the heteromodal areas of the brain showing the most prominent changes.[40] The value of graph theory measures in predicting disease progression has also been investigated in PD. Longitudinal analysis over a period of four years revealed progressive changes in functional brain network organization that were associated with increasing cognitive and motor impairments.[41] Finally, several key pathways in the limbic system, basal ganglia, and sensorimotor circuits have shown a reduced pattern of communication in early PD, providing support to the presence of disconnectivity mechanisms in motor as well as non-motor circuits since early disease stages.[43]

In conclusion, the assessment of brain connectivity and organization has provided an important insight into the underlying mechanisms of PD. Rather than targeting a few isolated brain areas, PD may be associated with dysfunction in large brain networks, which would explain the heterogeneity in motor and non-motor symptoms.[4] Hence, network measures hold promise as markers of disease progression in PD that may capture better the complexity of the disease.

Premotor and early PD: insight from functional brain assessment

Starting from the evidence that about 50% of nigrostriatal neurons are already lost when PD is diagnosed because of onset of motor symptoms, and from the notion of the high prevalence of some symptoms in the clinical history of PD patients before the appearance of motor symptoms, the concept of a premotor (or prodromal) stage of PD has emerged in the last years.[44] These symptoms are many but those with the highest prevalence are rapid eyes movement (REM) sleep behavior disorder (RBD), constipation, depression, hyposmia, and excessive daytime sleepiness which have been reported to antedate the motor stage even by decades. An effort to intercept PD in this prodromal stage would be of para-

mount importance to test potentially neuroprotective drugs and interventions in order to delay the course of neurodegeneration. It is a hypothesis that previous clinical trials devoted to demonstrate the efficacy of drugs to slow down the course of neurodegeneration in PD failed just because they were used in a too advanced stage of nigrostriatal neurodegeneration. Unfortunately, these symptoms are either easy to be detected in the general population but relatively unspecific (such as hyposmia, depression, constipation, excessive daytime sleepiness) or very specific but difficult to be demonstrated (RBD). However, the positive likelihood ratio of RBD to predict PD in the following five years is 30 to 70 times higher than the ones of each of the other symptoms.[45] Therefore RBD represents the most proficient starting point in the attempt to detect prodromal PD patients. RBD were firstly described by Schenck *et al.*,[46] have a prevalence of about 0.5% among the general population and are characterized by complex and often violent behaviors during REM sleep, and by the absence of physiological muscle atonia during REM sleep (so that the patient enhacts dreams with possible harmful behavior to the bedpartner and him/herself). The main practical issue with RBD is that they need to be demonstrated by means of polisomnography (PSG), a complex, time-consuming, costly and not largely available tool. In this context, REM sleep without muscular atonia can have the same meaning but is shown only by PSG. However, RBD may be suspected based on a careful clinical interview with the bedpartner and thanks to structured questionnaires, such as the Mayo Sleep Questionnaire (MSQ),[47] which has been validated *versus* PSG, reporting a very high accuracy. With this questionnaire the main problem arises when the patient has no bedpartner since he/she is often unaware of RBD, which happens at least in the 10% of cases. Falls from the bed during sleep or trauma deriving from violent movements are the only way to suspect RBD in a patient sleeping alone while amateur videocamera to be put in the bedroom is an option to be further explored. The MSQ can be used in a clinical setting to screen patients to submit to PSG for RBD confirmation.

In the following paragraphs we discuss developments in molecular imaging studies in pre-motor PD and RBD, including brain DAT SPECT, ^{18}F-Fluorodeoxyglucose PET (FDG-PET) and ^{123}I Metaiodobenzylguanidine (MIBG) cardiac planar scintigraphy.

Dopaminergic imaging

In RBD patients the demonstration of nigrostriatal impairment by means of presynaptic dopaminergic brain imaging (with SPECT or PET) strongly increases the probability of prodromal PD. It has been calculated that such kind of patient has a higher than 90% likelihood to have prodromal PD, irrespective of his/her age.[45] This had led to recommend the use of dopamine transporter (DAT) imaging in RBD patients with a 1st class level recommendation in a recent review [48] although the big issue remains of what to communicate to these patients, since disease modifying therapies are lacking yet, and overall how to do it.[49] However, the use of DAT imaging has not been approved by regulatory Agencies to be used in this context, and should remain till now in a research scenario. Another point deserving attention is that specific binding ratio (SBR) on DAT SPECT should not be simply 'reduced' but be lower than -2 Standard deviations below the age-adjusted values of a healthy control population.[45]

This raises the issue of need for a semi-quantification tool and for normal controls (see below for further details). Iranzo *et al.*[50] studied a group of RBD patients with DAT SPECT and followed them for three years with serial examinations. As compared to healthy controls they found a progressive decrease of SBR values in RBD patients. Moreover, the three RBD patients with the lowest SBR values at baseline developed PD within three years. Although the decrease of putamen and caudate SBRs were similar, at least at 18 months the caudate decrease seemed to be stronger than putamen one. Some years later it has been hypothesized that nigrostriatal deafferentation at caudate level is especially evident in RBD patients.[51] In fact, patients with idiopathic RBD (iRBD) showed higher putaminal SBRs but lower caudate SBRs than PD patients without RBD, and similar caudate SBRs as PD patients with RBD.

Additionally, assessing brain function by means of molecular imaging in early and premotor PD certainly has diagnostic value but could also disclose prognostic value. In a group of fifty-four patients with de novo PD undergoing a 5-y follow-up (in average) we found that DAT SBRs at caudate level and the mean frequency of quantitative electroencephalography have the highest predictive power toward cognitive worsening. This predictive value exceeded 80% for each of the two modali-

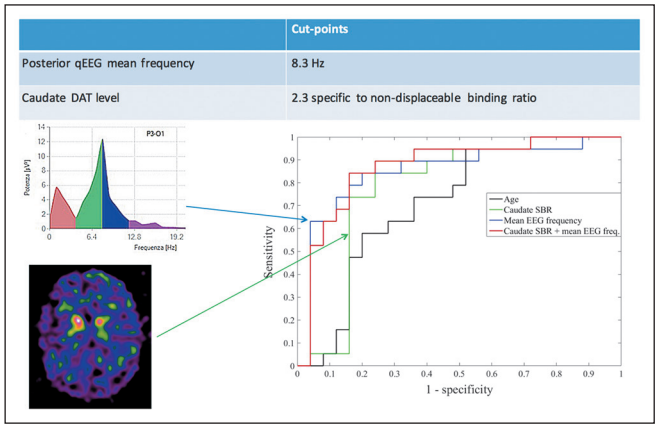

Figure 2.—Prediction of cognitive worsening in a group of de novo PD patients undergoing DAT SPECT with I-123 Ioflupane and quantitative electroencephalography. Each of the two modalities predicted (cross-validated) cognitive worsening with >80% accuracy.[52]

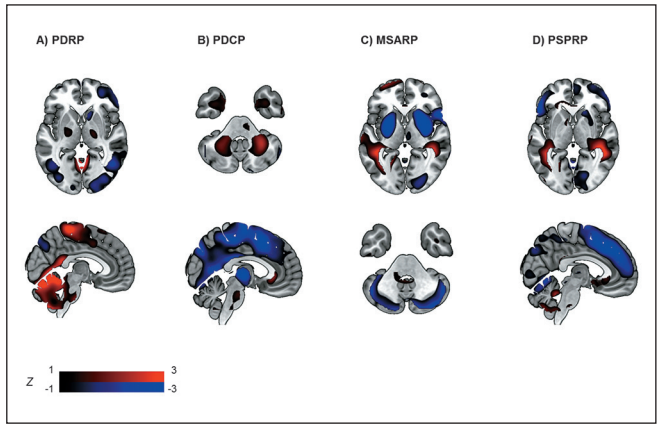

Figure 3.—Disease-specific brain metabolic patterns in parkinsonian disorders identified with SSM/PCA. Visualization of (A) the PD-Related Pattern (PDRP), (B) the PD Cognition-related Pattern (PDCP), (C) the MSA-related pattern (MSARP), and (D) the PSP-related pattern (PSPRP). All patterns are displayed as z-maps, thresholded at $|z > 1|$ (as indicated by the color bar), overlayed onto an MRI T1 template. Relative hypermetabolism is denoted by red; relative hypometabolism is denoted by blue. This research was originally published in Meles *et al.*[65]

ties, a higher figure than reached by neuropsychological tests, age, and clinical classification (Figure 2).[52]

FDG-PET: spatial covariance patterns

Many neurodegenerative diseases are associated with specific patterns of abnormal cerebral glucose metabolism measured by FDG-PET. Such patterns can be extracted from FDG-PET data with scaled subprofile model and principal component analysis (SSM PCA), a technique first described by Moeller *et al.,*[53] and further developed and validated by Eidelberg and colleagues.[54, 55] Application of SSM PCA to resting state FDG-PET data in PD patients has identified a highly reproducible brain pattern, the PD related pattern (PDRP),[56-58] characterized by relatively increased metabolism in the pallidum, thalamus, pons and cerebellum, and by relatively decreased metabolism in premotor and parietal association regions. The expression of such a pattern can be computed in the FDG-PET scans of new individuals,[59] yielding a single score per scan (the subject score). PDRP subject scores are significantly (but moderately) correlated with motor symptoms and DAT binding.

Capitalizing on the application of these 'disease-specific related patterns', the presence of certain FDG-PET patterns has also been explored in patients with multisystem atrophy (MSA), and patients with progressive supranuclear palsy (PSP) (Figure 3).[60-62] The application of these patterns to PD and atypical parkinsonisms has resulted in the development of a highly accurate method for discriminating between these differential diagnoses.[63, 64] The potential of FDG-PET has been recently reviewed by Meles *et al.*[65]

Using a similar method, a spatial covariance pattern specific for cognitive decline in PD was identified in two cohorts. The first PD cognition-related pattern (PDCP) was described by Huang *et al.* in an American cohort.[66, 67] We were able to validate this 'original PDCP' in a cohort of non-demented Dutch PD patients, and also re-derived a PDCP. Expression of the two PDCPs was strongly correlated to each other, with notable hypometabolism in medial frontal and parietal regions, and relative hypermetabolism in the cerebellum being prominent features of both PDCPs. Differences between the patterns may be accounted for by the different neuropsychological tests administered to define the respective non-demented, cognitively-impaired PD cohorts. Additionally, it was found that both the American and Dutch PDCP scores were higher in patients with mild cognitive impairment, compared to those with a normal neuropsychological evaluation. Finally, PDCP subject scores were significantly correlated to perfor-

mance on neuropsychological tests of executive and memory function.

The exact origin of the cognitive decline in PD patients is not entirely understood, and likely represents the result of a heterogeneous set of processes including cholinergic and dopaminergic denervation, as well as Lewy body and plaque formation.[68] Nevertheless, the metabolic alterations underlying cognitive decline in PD appear to be similar across PD populations, signifying that the PDCP is a reliable progression marker of cognitive dysfunction in PD.

As improved treatment options for the motor symptoms of PD become more available over time, cognitive decline is gradually becoming known as one of the major determinants of overall quality of life and functional outcome in PD patients.[69-71] Because of this, and considering that PD patients are 4-6 times more likely to develop dementia than the general population over the course of the disease,[72, 73] the need for a stable biomarker of cognitive impairment in non-demented PD patients has increasingly become more relevant. The PDCP may be useful in the context, but additional, longitudinal studies are needed to establish its utility.

FDG-PET spatial covariance patterns in RBD

In RBD, PDRP expression has also been studied as a biomarker for phenoconversion to PD. Three studies have shown that PDRP subject scores are significantly higher in PSG-confirmed RBD patients compared to controls.[65, 74, 75] In each of these studies, PDRP subject scores were lower compared to patients with established PD or DLB. In a cohort of 20 patients, high baseline PDRP expression (>1) in brain perfusion imaging (^{99m}Tc-ECD SPECT) was more likely in iRBD patients (N.=8) who developed PD or DLB 4.6±2.5 years after scanning.[76] In contrast, RBD patients who later developed MSA did not express the PDRP.[76] PDRP expression may provide complementary information to other markers such as DAT binding and olfaction.[65] In contrast to olfaction,[77] the PDRP is a progression marker.[66] Moreover, PDRP expression is useful in the differential diagnosis of parkinsonian disorders,[64] whereas DAT imaging is not.[78]

The metabolic profile of idiopathic RBD itself has also been studied with SSM PCA. In 2014, Wu *et al.*[75] described an RBD-related metabolic brain pattern (RBDRP) in FDG-PET scans of iRBD patients and controls using SSM PCA. This RBDRP featured relative hypometabolism in the posterior temporal cortex at the temporo-occipital junction (middle temporal gyrus), as well as in the lateral, polar (*i.e.* calcarine), and mesial (*i.e.* lingual) occipital lobes; there was also relative hypermetabolism in the supplemental motor cortex, middle cingulate, orbitofrontal cortex, superior frontal gyrus and putamen. Wu's group demonstrated that the RBDRP was significantly expressed in RBD patients (whole-brain analysis) and in the least affected hemisphere of PD patients with early, unilateral PD. However, RBDRP subject scores were lower in patients with more advanced PD. This led their group to conclude that the RBDRP must have altered metabolism in regions specific to early or prodromal stages of PD: "the RBDRP is perhaps relevant only for prodromal RBD cases and likely breaks down with disease progression."

We recently studied the RBDRP in an independent cohort of RBD patients (N.=21) and controls (N.=19) (Meles *et al.*, manuscript submitted). In keeping with Wu's 'original' RBDRP, our pattern had relative hypermetabolism of the thalamus, hippocampus, and pons, and relative hypometabolism of the parietal, temporal and occipital cortices.[75] In addition, in contrast to Wu's pattern, our RBDRP had relative hypermetabolism in the putamen, pallidum, and cerebellum.[74] In our analysis, the putamen and pallidum were not stable on bootstrap resampling, but did nevertheless contribute to the RBDRP score; this may have been related to the patients' level of dopaminergic damage. As dopaminergic imaging was not performed in Wu's study, it is unknown how many patients in their RBDRP identification cohort may have had presynaptic dopaminergic denervation. Additionally, our RBDRP revealed relatively decreased metabolism of the middle cingulate, while in Wu *et al.*, the middle cingulate appeared to have a relatively increased metabolism. Finally, relative hypometabolism in the parietal cortex was a prominent feature of our RBDRP, whereas this was not seen in Wu's pattern. Overall, our RBDRP is strikingly similar to the PDRP, in contrast to Wu's RBDRP (correlation coefficient r=0.94 *versus* r=0.39).

Ultimately, our RBDRP scores correlated strongly with PDRP scores. However, Arnaldi *et al.* showed that PD patients with RBD had more severe relative hypometabolism in posterior association cortices than

PD patients without RBD,[79] thus standing for an even more severe clinical phenotype in patients with both PD and RBD than in those with PD only. Additionally, bilaterally-affected PD patients had slightly higher RBDRP scores than unilaterally-affected patients, but this did not reach statistical significance. However, RBDRP scores were significantly higher in PD patients with MCI than in those with normal cognition; additionally, most of the PD-MCI patients also had probable RBD and may represent a more severe subtype of PD. We concluded that RBDRP is likely a predecessor of the PDRP and, similarly to the PDRP, its expression increases with disease progression.

It is possible that some of the differences in pattern-generation and subsequent RBDRP scores between our study and Wu *et al.* one, may be accounted for by unintentional differences in our respective RBD patient samples. As RBD represents a prodromal stage of Lewy-body diseases (LBD), it is unknown to which alpha-synucleinopathy (PD, DLB, or MSA) each patient will ultimately convert, and indeed 10% may never convert.[77] It is also worth noting that prodromal MSA patients may not have a high PDRP expression at baseline, and it is therefore possible that they may not have high RBDRP expression either.[65, 74] In addition to the possibility that our RBD cohort surreptitiously included more non-converters or prodromal MSA patients than Wu's group, it is also possible that the respective patient groups may have been at different disease stages overall. Most disease patterns are made up of the first or first two PCs.[56-58] The fact that Wu's group was able to identify their RBDRP using only the first PC, while our pattern was made up of PCs 4 and 5 may lend credence to this theory, as the use of lower eigenvalues indicates that the variance among our RBD subjects was greater than the overall difference between RBD patients and controls. As all of our RBD patients were premotor, and the majority had normal dopamine transporter scans, conceivably some patients may have had brain metabolic profiles closer to those of healthy controls than to PD patients. Therefore, the first few PCs in our dataset likely describe normal resting-state brain function; this seems to be supported by Spetsieris *et al.*, where PCs 1 and 2 described among several cohorts of healthy controls were very similar to our own.[55] In contrast, if the patient cohort in Wu *et al.* were indeed in a more advanced stage of disease, it would make sense that there would be a stronger disease-related signal in the the FDG-PET data which would subsequently be represented by the first PC.

The main takeaway from this detailed examination of differences between ours and Wu's RBDRP studies is that there are a number of potentially confounding factors which must be taken into consideration. This is particularly true when delving into the topic of prodromal metabolic brain patterns, where it may not be possible to delineate the patient's global condition as strictly as in a progressed disease state.

Another study by our group examining PDRP scores among RBD patients further supports the notion that RBD is an early form of LBD.[80] It is the first attempt of its kind to study the correlation between PDRP scores, DAT SPECT, and olfaction in RBD patients. Patients' level of olfaction was tested using Sniffin' Sticks and divided into two groups, those with total olfaction scores (TDIs) <18 and those with TDIs ≥18, in line with a previous study by Mahlknecht *et al.* which showed that a baseline TDI score of <18 was associated with an elevated risk of conversion to PD or DLB within five years of follow-up.[81]

Most notably, it was found that over half of the RBD patients already had PDRP scores in the range of PD patients at baseline. Overall, the PDRP scores seen in this study's RBD patients were significantly elevated as compared to healthy controls, but lower than in PD and especially DLB patients (Figure 4). TDIs <18 and abnormal DAT-binding both separately associated with increased PDRP scores in RBD patients, with borderline significance; previous studies have shown that DAT-binding tends to be only modestly correlated with PDRP scores.[62, 63, 82] This may be in part due to a non-dopaminergic origin of the PDRP.

It should be considered, however, that PDRP on FDG-PET is a dynamic phenomenon along the course of a disease lasting decades, some before the onset of motor symptoms and the rest in average two decades later on. As FDG-PET reflects synaptic density and function it is obvious that a given pattern could change as a function of the time point when the examination is performed. This extreme sensitivity of FDG-PET for the functional asset of the brain might explain at least some of the discrepancies among studies by different groups.

There was also a subgroup of patients who had PDRP scores in the range of PD patients, but with normal

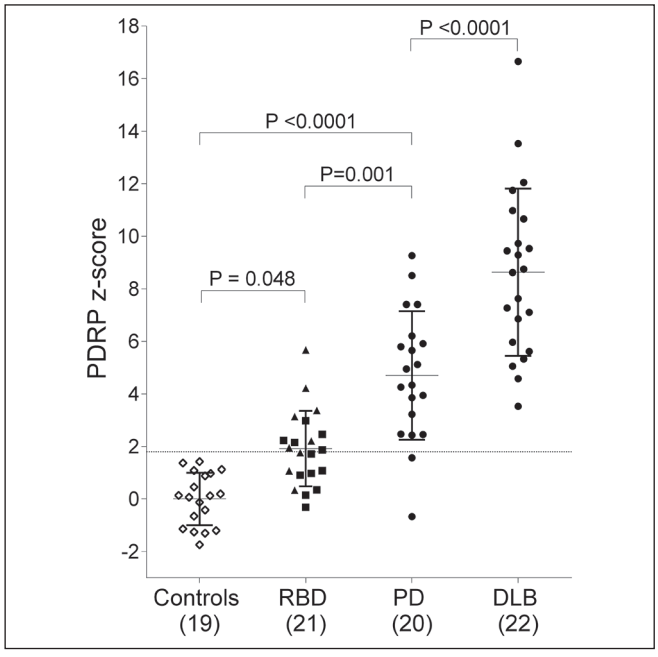

Figure 4.—PDRP z-scores across groups. PDRP expression was calculated in all groups and z-transformed to the healthy controls. PDRP expression z scores were compared across groups with a 1-way analysis of variance. *Post-hoc* comparisons were Bonferroni-corrected.
The dashed line (z=1.8) indicates the cutoff for PDRP expression. Triangles indicate RBD subjects with abnormal DAT scans. Squares indicate subjects with normal DAT scans.
With permission from Wiley Publishing Meles *et al*. FDG PET, Dopamine Transporter SPECT, and Olfaction: Combining Biomarkers in REM Sleep Behavior Disorder; Movement Disorders (MDS).

DAT-binding. This could potentially indicate that these patients may have a greater likelihood of converting to dementia with Lewy bodies (DLB) as opposed to PD, as one study has shown that early DLB patients may initially have negative DAT scans.[83] There was also some association between putaminal DAT-binding and olfaction, however it did not reach statistical significance. The exact relation of olfaction to the other factors may be difficult to pinpoint, as olfaction is not a progression marker. This lack of significant correlation could also indicate that PDRP scores, DAT-binding, and olfaction all provide complementary information.

Taken together, the data illustrate that from a global metabolic point of view, the brains of iRBD patients are collectively on the path towards developing LBD; and the closer these patients get to imminent conversion, the higher their expression of the pattern becomes. Moreover, a similar but more-pronounced pattern of relative hypometabolism in the posterior temporo-parieto-occipital cortex, and relative hypermetabolism in the premotor and supplementary motor cortices, basal ganglia, and medial temporal lobe structures has been reported in de novo PD patients with RBD as compared to PD patients without RBD.[79]

Non-dopaminergic imaging

The hypometabolism in posterior association cortex has been interpreted as a consequence of the profound cholinergic deficit observed in PD patients with RBD as compared to those without RBD.[84] The cholinergic deficit seems an early phenomenon along the story of PD and it has been recently detected also at the level of colon and small intestine in PD patients by means of PET and an acetylcholinesterase inhibitor labelled with C-11 (C-11 Donepezil).[85] Since the current hypothesis of PD pathogenesis admits that the disease begins in the intestine and then ascends to the brain via the visceral nervous system and the vagal nerve, the role of these new PET tools could be of crucial relevance in the future to intercept the very early stages of PD pathophysiology. At present, even if cholinergic intestinal or brain PET is unavailable for clinical use, one could make inference of the status of brain cholinergic tone thanks to the work of Muller *et al.*[86] who found the clinical correlates of cortical cholinergic deficit and of the combined cortical-thalamic cholinergic deficit in PD patients. Indeed, cortical cholinergic deficit is predicted with 80.7% accuracy by the combination of slowness of walking and impaired score on the MOCA cognitive test whereas cortico-thalamic cholinergic deficit is predicted with 81.1% accuracy by the combination of RBD and history of falls. The presence of these symptoms may thus help the clinicians to speculate on the neurochemical basis of deficit of their patients and possibly to guide symptomatic treatment.

The use of I-123 MIBG scintigraphy has shown that the majority of iRBD patients also have myocardial noradrenergic deafferentation.[87, 88] Subsequent studies have also demonstrated that even PD patients without orthostatic hypotension do have impairment on I-123 MIBG scintigraphy that can detect visceral denervation before onset of dysautonomic symptoms,[89] but also that not all PD patients disclose I-123 MIBG impairment.[90] These two findings could be only apparently in disagreement one another. Indeed, the scenario of differ-

ent PD phenotypes is emerging. On the one extreme the malignant phenotype is characterized mainly by RBD, orthostatic hypotension and MCI, on the other extreme are those patients without any of these issues, *i.e.*, those with mainly motor symptoms/signs only, and in the middle between are those with one-two of them, representing the "intermediate" phenotype.[91] Cardiac adrenergic denervation might be typical of the malignant and intermediate phenotypes whereas at least some of the mainly motor forms could have normal cardiac innervation. In a principal component analysis of patients with de novo PD, a normal MIBG scintigraphy stands alone and very far from a positive cardiac scintigraphy that instead is close to RBD, MCI, constipation, urinary dysfunction and excessive daytime sleepiness.[90] Moreover, some patients with multisystem atrophy-Parkinsonism can disclose impairment on MIBG scintigraphy due to sparse Lewy bodies along the post-ganglionic nerve to the heart.[92] These are the reasons why, although rather accurate in detecting LBD, MIBG cardiac scintigraphy cannot probably be used as the only examination to confirm/rebut a clinical diagnosis of PD.[93]

Test-retest reproducibility and harmonization

The use of presinaptic dopaminergic imaging in patients with suspected prodromal PD has been attempted also in patients with idiopathic hyposmia who were shown to have decreased SBRs both at caudate and at putamen level on repeated DAT SPECT after two years.[94] Three out of the four patients developing PD in a 2-year period disclosed a SBR reduction of about 20%, but unbelievably one disclosed a 40% increase. Looking at plots of the Ponsen *et al.* paper, [94] it appears a very high variability of SBRs at repeated *versus* baseline examination, with increases and decreases within the 40% from baseline. If DAT expression decrease can be expected, it is difficult to understand the increases that exceed the 5-10% that could be due to chance, test-retest reproducibility, or some underlying physiological changes. This raises the issue of how SBRs are measured, an issue that is still unresolved, as it has been shown that the average test-retest variability exceeds the 10%, irrespective of the measure adopted among MRI-guided VROI, atlas-guided VROI, and hand VROI drawing.[95] However, reproducibility of semi-quantitative assessment of brain imaging is not limited to dopaminergic SPECT imaging. For instance, a similar problem has been met by the PET core lab of the AD Neuroimaging Initiative when trying to apply semi-quantitative evaluation based on SUVr computation in longitudinal ^{18}F-18 Florbetapir PET. They found a non-trivial number of patients whose amyloid load seemed to decrease with time, without any treatment.[96] In another experience applying different quantification tools to evaluate medial temporal atrophy, a part of AD patients had their hippocampus increased in volume with time, and they differed depending on the algorithm used for quantification.[97] More effort should be dedicated to optimization of semi-quantitative measures and of harmonization among centers, as it has been recently announced by the Strategic Research Agenda in Neurodegenerative Diseases (SRA-NED) Consortium (http://www.sra-ned.org/). Indeed, harmonization of image acquisition and data analysis remains challenging since biomarkers should be specific to the disease. As a result, an inadequate level of calibration protocols prevents comparisons of data from different studies and delays the use of biomarkers in clinical research and drug discovery, ultimately leading to uneven and suboptimal quality of care and the slowing of drug discovery.

This limitation could be less heavy for DAT SPECT with I-123 Ioflupane since an effort has been made by the European Association of Nuclear Medicine-Neuroimaging Committee (EANM-NIC) to harmonize acquisition, elaboration, and semi-quantification of the examinations across European centers. The EANM-NIC DAT initiative (ENC-DAT) led to the generation of precious calibration coefficients to harmonize acquisition with the most common gamma camera on the market [98] and of common rules to be followed in multicenter studies.[99] Also, normal reference values on a large cohort of normal individuals have been generated within the ENC-DAT study,[100, 101] including the use of original and free software.[102] Maybe the advent of new radiopharmaceuticals to image presynaptic terminals with PET technology (the F-18-9-Fluoropropyl-Dihydrotetrabenazine, a marker of dopamine vescicular transporter, is one of the candidates)[103] leads to a substantial improvement of test-retest data, partly overcoming the limitations of SPECT technology.

The PETMETPAT project, which falls under the umbrella of the EU Joint Programme – Neurodegenerative Disease Research (JPND), currently seeks to harmonize

brain ^{18}F-FDG-PET acquisition protocols across Europe as well. PETMETPAT is currently oriented specifically towards equilibrating PDRP scores derived from ^{18}F-FDG-PET scans done on various scanners at different centers; these PDRP scores are based on an established disease metabolic pattern quantified by spatial covariance analysis in PD patients as compared to controls.[54] A study by Ikari *et al.* has identified and explored similar harmonization problems with respect to AD, and concluded that the use of a Hoffman 3D Brain Phantom likely represents the best approach for equalizing image reconstruction protocols across different machines.[104]

A study by Tomše *et al.* showed that when employing different PET scan reconstruction algorithms, there was some overlap in PDRP scores between PD patients and controls. However, these reconstructions, which among other variables included varying conditions related to time-of-flight (TOF), point-spread function (PSF), and a spectrum of Gaussian filters, resulted in systematic shifts in scores which led Tomše's group to conclude that these factors did not significantly impede the ability to discriminate PD patients from healthy controls.[105] However, our own work examining the effects of various reconstruction methods on PDRP and Alzheimer's disease related-pattern (ADRP) scores shows that there can indeed be significant overlap between healthy controls and disease patients (PD and AD), making it difficult to always differentiate patients from controls; this effect is further amplified in intermediate disease states (such as in RBD and MCI, respectively).

Additionally, preliminary surveys of PETMETPAT participants have shown that in addition to relatively-straightforward scan reconstruction setting differences, there may also be significant human variations in factors such as ^{18}F-FDG uptake time, injected activity, and blood glucose levels which may unduly skew PDRP scores. All of this bolsters the urgency of harmonizing PET scan protocols, in particular as the use of brain metabolic disease-related pattern scores gain popularity in clinical and research practice, as evidenced by the rapid expansion of databases such as the GLIMPS Project at the University Medical Center Groningen (http://www.glimpsproject.com/). To account for the effects of varying acquisition and reconstruction protocols, PETMETPAT will indeed use a collection of Hoffman 3D Brain Phantom scans gathered from various centers across Europe in an attempt to adequately harmonize FDG-PET brain scans.

However, it is also important to note that there may always be limitations to how well we are able to discern various groups of patients both from healthy controls and from each other using disease-related metabolic brain pattern scores alone. Even if FDG-PET scans are acquired in a uniform way from the same machine, there are an unquantifiable number of factors which may influence disease pattern scores in one way or another. For instance, in a recent study done by our group (Meles *et al.*, submitted) examining ADRP scores in an Italian cohort of MCI and AD patients based on an established AD-related metabolic brain pattern,[106] it was possible to discriminate MCI patients who ultimately converted to AD from healthy controls with a high level of sensitivity and specificity. However, it was much more difficult to differentiate MCI converters from non-converters. Of course, there is a possibility that some of the non-converters may yet end up converting to AD upon longer follow-up. But interestingly, it was found that for MCI non-converters with the highest ADRP scores, there was a borderline-significant correlation with having higher education; speculatively, this could point to the presence of a larger cognitive reserve in these subjects, with possible employment of compensatory networks helping to maintain a higher level of cognitive function despite regional hypometabolism.[107, 108] MCI non-converters were also the only subgroup of patients for whom age correlated significantly with ADRP scores. The conclusion is that all scanning protocols being equal, there may still always be confounding factors to prevent perfect separations between groups based on FDG-PET-quantified disease-related pattern scores alone.

Conclusions

To summarize, we can now image system dysfunction at molecular level already in the premotor stage of PD. RBD seems the most specific symptom preceding an alpha-synucleinopathy, although it must be stressed that a considerable part of patients do not develop PD but instead DLB. Moreover, some rare case also develops MSA. Combining PSG-proven RBD with impairment on presynaptic dopaminergic imaging strongly raises the risk to develop a LBD in the subsequent five years. However, this approach cannot be intended for clinical use yet, since more longitudinal data are needed, and be-

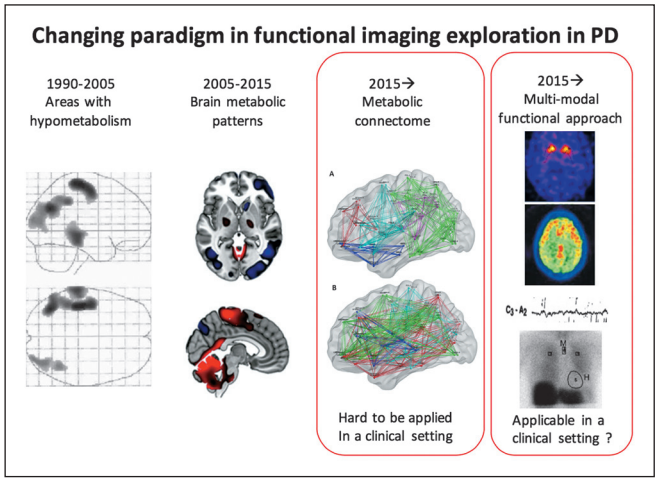

Figure 5.—Vignette on how functional imaging in PD is going to develop in the last years thanks to advanced computational approaches and to integrated multimodal imaging.

cause we actually do not have any neuroprotective drug. Both FDG-PET and I-123 MIBG scintigraphy may be useful to study RBD patients but again they are still in a research scenario and, on a clinical ground, they are better employed in the differentiation among Parkinsonian syndromes. Future development also includes advanced computational approaches, such as connectome investigation, and integrated multimodal imaging tailored to a specific clinical condition (Figure 5).

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