Title: Metabolic connectomics targeting brain pathology in dementia with Lewy bodies

Short title: FDG-PET connectomics in DLB

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Abstract

DLB is characterized by α-synuclein accumulation and degeneration of dopaminergic and cholinergic pathways. To gain an overview of brain systems affected by neurodegeneration, we characterized the [18F]FDG-PET metabolic connectome in 42 DLB patients, as compared to 42 healthy controls, using SICE method and graph-theory. We performed whole-brain and anatomically-driven analyses, targeting cholinergic and dopaminergic pathways, and the α-synuclein spreading. The first revealed substantial alterations in connectivity indexes, brain modularity and hubs configuration. Namely, decreases in local metabolic connectivity within occipital cortex, thalamus, and cerebellum, and increases within frontal, temporal, parietal and basal ganglia regions. There were also long-range disconnections among these brain regions, all supporting a disruption of the functional hierarchy characterizing the normal brain. The anatomically-driven analysis revealed alterations within brain structures early affected by α-synuclein pathology, supporting Braak's early pathological staging in DLB. The dopaminergic striato-cortical pathway was severely affected, as well as the cholinergic networks, with an extensive decrease in connectivity in Ch1-Ch2, Ch5-Ch6 networks and the lateral Ch4 capsular network significantly towards the occipital cortex. These altered patterns of metabolic connectivity unveil a new in vivo scenario for DLB underlying pathology in terms of changes in whole brain metabolic connections, spreading of α-synuclein, and neurotransmission impairment.

Keywords: *Brain Connectivity, Graph theory, Neurotransmission, SICE, Synucleinopathy.*

Introduction

Dementia with Lewy Bodies (DLB) is the second most common neurodegenerative dementia, characterized by a progressive and fluctuating cognitive decline, variably accompanied by extrapyramidal features, rapid eye movement sleep behaviour disorder (RBD), and visual hallucinations.[1]

DLB is linked to a disturbance of protein metabolism, producing an abnormal accumulation of α-synuclein in the brain.[2,3] It has been proposed that *α*-synuclein diffuses following a prion-like mechanism causing synaptic dysfunctions, and progressive neuronal death.[4,5] Braak and colleagues formalized the topographical progression of *α*-synuclein pathology in Parkinson's disease (PD) and DLB, from brainstem to neocortical structures, among cells sharing common features.[6,7]

Accumulation of α-synuclein is the key event causing synaptic dysfunction and leading to neurodegenerative symptoms in both DLB and PD.[8] The link between pathology and neurodegeneration is mediated by the *metabolic* pathways involved in neuronal function and bioenergetics.[9–12] In DLB there is a consistent evidence for a specific dysfunctional pattern, characterized by widespread reductions of metabolism in occipital, parieto-temporal and, to a lesser extent, frontal cortex, supportive for clinical diagnosis.[1,13]

The neurodegeneration of dopaminergic and cholinergic neurotransmission systems is a relevant aspect in DLB pathogenesis, as shown by *post-mortem*[14–16] and *in vivo*[17,18] brain studies, and is the basis for the major clinical features.[1] The accumulation of α-synuclein is associated with cholinergic[14,19], and nigrostriatal dopaminergic[8,20] deficits.

Specific brain circuits, as shown in fMRI studies, are disrupted in the different neurodegenerative conditions, in keeping with the principle of selective neuronal vulnerability, according to which specific neuronal populations die, whereas other are

resistant to neurodegeneration.[21] Previous functional Magnetic Resonance Imaging (fMRI) studies of brain connectomics in neurodegenerative diseases, showed a reduced connectivity in the default mode network in Alzheimer's Disease (AD), whereas the behavioural variant of frontotemporal dementia has been associated to a disruption of the anterior salience network. An impairment of thalamo-cortical and cerebellar-thalamo-cortical networks was also reported in PD and in progressive sopranuclear palsy, respectively (cfr. [22]). A functional network perspective was also adopted in some fMRI studies on DLB showing mixed results regarding DLB connectivity profile.[23–28]

[18F]FDG-PET is considered an index of the integrated local synaptic activity,[12] and its signal has also been associated to synaptic density.[11] [18F]FDG-PET represents a unique tool for capturing the heterogeneous events that contribute to synaptic dysfunction and consequent neurodegeneration, such as altered intracellular signalling cascades, impaired neurotransmitter release, proteinopathies, and long distance disconnection.[9] Thus, it is widely recognised as a clinical tool for both early and differential diagnosis of dementia, by which synaptic alterations, occurring before neurodegeneration, can be identified.[9,29,30]

Neuropathological evidence has reported that the majority of neurodegenerative diseases are characterized by gradual spreading of pathology across specific brain regions, and this idea has led to consider them as "disconnection syndromes.[6,31,32] Studying neurodegenerative diseases with a network perspective may therefore help to explain the link between local vulnerabilities, long-range disconnection, and the effects of neuropathology on them.[22] The investigation of brain metabolic connectivity is based on the assumption that regions whose metabolism is correlated are functionally interconnected.[33] **This assumption has its foundations in a pioneering**

study, in which Horwitz and colleagues (1984) explored resting state brain metabolic connectivity in healthy subjects, demonstrating that the results obtained with metabolic connectivity are largely consistent with known anatomo-functional data.[33] Recent evidence indicates a close relationship between resting state functional brain connectivity obtained using fMRI, and glucose consumption, as measured by [18F]FDG-PET.[34,35] Riedl and colleagues (2016), simultaneously acquired fMRI and [18F]FDG-PET data for obtaining a novel measure suitable to calculate brain state effective connectivity[35]. The integrated brain metabolic [18F]FDG-PET with fMRI data provided directionality of brain signalling, since increases in local metabolism reflect an increase in afferent effective connectivity. This metabolic/functional connectivity approach supports the integrated value of these techniques.[35]

Crucially, no study has hitherto characterized the whole-brain and anatomically oriented metabolic connectivity in DLB using [18F]FDG-PET. To obtain the metabolic connectivity matrices, we used the sparse inverse covariance estimation (SICE) method.[36] This method is suitable for [18F]FDG-PET studies, since it allows reliable estimation of inverse covariance even with relatively small sample size.[36] Here, we aimed at identifying the brain metabolic connectivity changes in DLB that can shed light on the relationship between synaptic dysfunctions and the underlying pathology associated to DLB.[21,37] We tested a comprehensive model for altered brain connectivity in DLB, by considering connectivity in the whole brain and neurotransmission pathways, following the increasing evidence that functional connectivity and network metabolic activity are sensible to changes in neurotransmission.[38–40] We focused on the dopaminergic and cholinergic systems in order to explore the effects on metabolic disconnections and connectivity reconfiguration in each of these pathways, which

represent the major biochemical neuropathology. In addition, we analysed metabolic connectivity of brain regions affected by α-synuclein spreading, with the aim to *in vivo* explore and support the Braak's staging hypothesis to DLB condition.[6,7]

Materials and Methods

Subjects

DLB patients were retrospectively collected from the clinical and imaging database of the San Raffaele Hospital, Milan. Expert neurologists performed the final diagnosis, considering full neurological information and follow-up, neuropsychological assessment, CT or MRI, [18F]FDG-PET scans and the CSF measures, including CSF Aβ42, t-Tau, and p-Tau levels. Patients showing cerebrovascular abnormalities on MRI or CT were excluded from the analyses.

As supporting information for the diagnosis, we used a [18F]FDG-PET Statistical Parametrical Mapping (SPM) t-map comparing every single individual with a standardized healthy control group,[41] and we specifically looked for the pattern of hypometabolism in the medial and/or lateral occipital cortex, which is considered the hallmark of DLB, accompanied by temporo-parietal and frontal cortex hypometabolism [1,41,42] (see Supplementary Figure S1).

According to the diagnosis at the clinical follow-up, we identified 42 out of 83 patients fulfilling consensus criteria for DLB,[1] and not showing any neurological and psychiatric comorbidities. The group of patients was composed of 27 males and 15 females, with a mean age±SD of 72.26±6.74 years. 71.43% of DLB patients presented parkinsonism,

64.29% RBD, and 59.52% visual hallucinations. The DLB group had a mean disease duration±SD of 2.14±1.4 years.

We selected 35 cognitively normal healthy control (HC) subjects from the control database of Nuclear Medicine Unit, San Raffaele Hospital, Milan. In order to obtain equal sample size with the DLB group and to avoid inter-scanner variability, we added 7 FDG PET images from the Alzheimer's Disease Neuroimaging Initiative (ADNI) database (adni.loni.usc.edu). The final group of 42 HC comprised 22 males and 20 females, matched for age, with a mean age±SD of 72.10±5.93 years. Age and gender between DLB and HC groups did not differ (F=0.14, p=0.906; X2=1.224, p=0.268).

HC and DLB patient studies performed in Milan were approved by the San Raffaele Hospital Medical Ethics Committee. Both groups provided written informed consent, following detailed explanation of each experimental procedure.

ADNI controls gave written informed consent at the time of enrolment for data collection and completed questionnaires approved by each participating sites Institutional Review Board (IRB).

The protocols conformed to the ethical standards of the Declaration of Helsinki for protection of human subjects.

[18F]FDG-PET image acquisition and reconstruction

The [18F]FDG-PET scans of the 42 DLB patients and 42 HC were acquired using the same Discovery STE PET (3.27 mm thickness; in-plane FWHM 5.55 mm) manufactured by GE Healthcare.

The 77 (35 HC and 42 DLB) [18F]FDG-PET acquisitions performed at the Nuclear Medicine Unit, San Raffaele Hospital conformed to the European Association of Nuclear Medicine guidelines. [43] Static emission images were acquired 45 min after

injecting 185–250 MBq of [18F]FDG via a venous cannula. This post-injection time interval allows to obtain an equal distribution of the tracer across the entire brain, with negligible blood flow-dependent differences, thus achieving an optimal signal-to-noise ratio.[44] The duration of scan acquisition was of 15 min.

The 7 steady-state emission images obtained from the ADNI dataset were acquired 30 min after injecting approximately 185 MBq of [18F]FDG via a venous cannula, with a scan acquisition duration of 30 minutes. After realignment to correct for eventual inter-frame motion, the last three frames lasting 5 minutes each were combined to obtain a single 15-minute static image representing the distribution during the whole acquisition time. In this way, the uptake time of ADNI images was uniformed to the uptake time of San Raffaele images.

The ADNI was launched in 2003 as a public-private partnership, led by Principal Investigator Michael W. Weiner, MD, with the primary goal of testing whether serial magnetic resonance imaging (MRI), positron emission tomography (PET), other biological markers, and clinical and neuropsychological assessment can be combined to measure the progression of mild cognitive impairment (MCI) and early Alzheimer's disease (AD). For up-to-date information, see www.adni-info.org.

In this study, uniform reconstruction protocols were applied. In particular, the use of the ordered subset-expectation maximization algorithm, CT attenuation correction procedures. A rigorous quality control process was performed to check for major artefacts in PET raw images, including defective image uniformity and orientation, or attenuation correction due to a mismatch between CT and PET images.

Metabolic connectomics

[18F]FDG-PET image pre-processing

All scans underwent general pre-processing procedures, using the MATLAB (http://it.mathworks.com/products/matlab/) (Mathworks Inc., Sherborn, Mass., USA) based software SPM5 (http://www.fil.ion.ucl.ac.uk/spm/software/SPM5/). In details, each image was first normalized to a [18F]FDG-PET specific template registered to the Montreal Neurological Institute (MNI) standard space, developed by our group,[45] using the default SMP5 bounding box, and with an isotropic voxel size of 2 mm. The specific [18F]FDG-PET template provides high levels of normalization accuracy, and it reduces random effects due to noise.[45] All the normalized PET images were spatially smoothed with an 8 mm isotropic 3D Gaussian FWHM kernel. Moreover, every image was proportionally scaled to its global mean,[46] in order to account for between-subject uptake variability,[47] thus obtaining higher signal-to-noise ratio compared to other available scaling methods (e.g. cerebellar reference area).[48]

Node selection and ROIs definition

We created the *whole-brain metabolic connectivity matrix* (i.e. the full-matrix), considering cortical, subcortical, cerebellar, and brainstem regions (121x121 ROIs), with the objective to cover the whole brain structures. These regions represented the basic units of the network, i.e. the nodes. There were 92 nodes for cortical and subcortical structures, 26 nodes for the cerebellum, and 3 nodes for brainstem (Supplementary Table S1A).

Moreover, another analysis was performed according to Huang and colleagues (2010), and subdividing the full-matrix into 12 sub-matrices, representative of larger brain regions (see Supplementary Table S1B).

In addition, we performed a set of spatially-anatomically driven connectivity analyses, addressing neural networks related to DLB pathology. These included the cholinergic[49–51] and dopaminergic[52,53] neurotransmission systems, as well as the spreading of α-synuclein accumulation in the brain according to Braak's stages progression.[6,7]

As for the *α-synuclein analysis*, we considered Braak stages 1 to 4.[6] Braak's staging was originally proposed for Parkinson's disease and subsequently also for DLB,[7] supported by evidence show consistent data in the spreading of *α-synuclein* pathology.[54,55] We constructed the α-synuclein network considering medulla oblongata and pons, for stages 1-2, midbrain, hippocampus, parahippocampal gyrus, ventral striatum and amygdala for stages 3-4.[6] Medulla and pons contain grey matter nuclei early affected by α-synuclein pathology, i.e. the dorsal IX-X motor nuclei, intermediate reticular zone, caudal raphe nuclei, gigantocellular reticular nucleus and coeruleus/subcoeruleus complex[6] (Supplementary Table S2).

Concerning the *dopaminergic system* anatomically-driven analysis, we created two different networks representing the striato-cortical and meso-limbic pathways. The striato-cortical network consisted of dorsal caudate and dorsal putamen, frontal premotor, motor, executive dorsolateral frontal regions, and somatosensory cortex; the meso-limbic network consisted of ventral striatum, ventral and medial frontal areas, anterior and middle cingulate cortices, as well as the amygdala and parahippocampal cortex[52,53] (Supplementary Table S3).

For the *cholinergic system analysis*, we considered six different cholinergic pathways[49–51]. The first network consisted of the brain regions innervated by Ch1 and Ch2 nuclei of the forebrain, namely the bilateral hippocampus and hypothalamus. The second network represented the pathway arising from Ch3 nucleus, that reach the olfactory and

parahippocampal cortices. The third, fourth, and fifth cholinergic networks were represented by the pathways originating from Ch4 nuclei. Namely, the third Ch4 medial pathway reaches cingulate, retrosplenial, and orbitofrontal cortices; the fourth Ch4 lateral perisylvian division joints olfactory and superior temporal cortices, plus the insula and the frontoparietal operculum; the fifth Ch4 lateral capsular division supplies the remaining frontal, parietal, temporal, and occipital cortices, as well as the amygdala. The cholinergic nuclei of the brainstem, i.e. the Ch5 pedunculopontine tegmental nucleus and the Ch6 laterodorsal tegmental gray of periventricular area, reach the thalamus, ventral and dorsal striatum, globus pallidus, and the brainstem reticular formation represented by pons, midbrain, and medulla oblongata (Supplementary Table S4).

In all the aforementioned pathways, we excluded the small output nuclei from which each molecular network originates (i.e. substantia nigra, ventrotegmental area, and Ch1, 2, 3, 4, 5, 6 nuclei), due to the limited spatial resolution of PET method and the lack of reference atlases for these regions.

All the ROIs used for the connectivity analyses were derived from AAL atlas [56], with the following exceptions: i) for the hypothalamus, we created spherical ROIs with a 5-mm radius centred at [±8 -4 -4 mm] MNI coordinates, according to [57]; ii) for the brainstem, we used ROIs available in WFUPickAtlas Tailarach Daemon Lobar Atlas; iii) for the dorsal and ventral striatum, ROIs were manually drawn in MriCron (www.mricro.com), using a standard high-resolution MRI T1 image as anatomical template, in accordance to the structural subdivision of the basal ganglia proposed by Tziortzi and colleagues.[58]

Furthermore, prior to the analyses, we verified that the volume of each ROI included

was not smaller than three times the FWHM of the scanner spatial resolution. This spatial resolution cut-off is considered to be the lower limit to avoid confounding effects such as blurring or spill-over (i.e. ROI partial volume effects).[59,60]

Construction of Brain Connectivity Matrices

We created whole-brain, dopaminergic, cholinergic, and α-synuclein Subject-by-Node/ ROI matrices for each group (HC, DLB). The matrices contained the regional cerebral metabolic values derived from each subject in each node/ROI. To obtain the metabolic connectivity matrices, we used the sparse inverse covariance estimation (SICE) method.[36] This method has been previously used in other [18F]FDG-PET studies, since it allows reliable estimation of inverse covariance even with sample sizes equal or even smaller than the number of nodes selected. This is crucial as in [18F]FDG-PET studies the sample size is the number of subjects and not the length of the time series, as in fMRI studies.[36] In order to apply SICE, we verified that data followed a multivariate normality distribution. Even though multivariate normality of cerebral metabolism data is usually assumed a priori,[36] we nevertheless verified it performing a Mardia test. Since the number of variables (i.e. nodes) was greater than the number of cases, we performed multiple multivariate normality tests on smaller random subsamples of nodes, so that the assumptions for the application of Mardia test were fulfilled. We found that both [18F]FDG-PET metabolic data of HC and DLB followed a multivariate normality distribution.

Using the GraphVar toolbox,[61] we obtained unweighted binary metabolic connectivity matrices from Subject-by-Node matrices. Unweighted binary metabolic connectivity matrices consist of zero and non-zero entries, respectively indicating the presence and absence of a significant partial correlation between two nodes (i.e. a functional

connection between the two nodes).[62] We computed metabolic connectivity matrices with different densities, representing different numbers of connections. As there is no gold standard for the number of connections to be selected,[63] we considered matrices with three different numbers of connections, that were proportional to the total number of nodes. For the whole-brain and cholinergic analyses, we used matrices with 200, 400, and 600 connections. Given the smaller total number of nodes in the other anatomically-driven analyses, we set matrices with 60, 120, and 180 connections for the dopaminergic system and matrices with 10, 20 and 30 connections for the α-synuclein network. Unweighted matrices were used for statistical testing, as reported below. Note that we set the number of connections to be the same for both groups. Setting a fixed threshold for both groups is a common strategy to compare the organization of connectivity between different groups, factoring out global connectivity differences (see [36]).

For each group, a summary whole-brain *semi-weighted* matrix was also obtained by summing up the three unweighted binary matrices with 200, 400, and 600 connections. The same procedure was applied to the binary matrices for dopamine, acetylcholine, and α-synuclein, in order to obtain semi-weighted matrices for each atlas at the three different densities. Note that when SICE is applied, the weight of the connections has to be interpreted as an ordinal measure.[36] Therefore, a single semi-weighted matrix containing "a-quasi measure" of strength (from 1 = lowest strength to 3 = highest strength) was obtained for each DLB and HC group. Semi-weighted matrices do not provide a numerical information on the strength of connections, but a useful visual representation of the distribution of connections according to their strength level. Semi-

weighted and unweighted metabolic connectivity matrices were represented in a 3D brain template using BrainNet toolbox (https://www.nitrc.org/projects/bnv/).[64]

Graph theory analyses

For the whole-brain full-matrix, we performed both a complex brain network analysis and a nodal analysis, using a set of functional connectivity indices.[65] For these analyses, we only considered the highest density network with 600 connections, in order to extract the best characteristic indices of network organization (see [63]). We computed average clustering coefficient (C) and modularity (Q) as measures of network segregation. A segregated network is characterized by the presence of densely interconnected groups of brain regions with specialized processing functions.[65,66] Global efficiency (E) and characteristic path length (L) were calculated as measures of network integration, accounting for the coordinate activity of distributed brain regions.[65,66] We also considered the small-worldness (S) index, representing the relationship between segregation and integration.[65] S was computed adopting the formula $S=(C/C_{rand})/(L/L_{rand})$ (cfr. [65]). C_{rand} and L_{rand} correspond to the mean clustering coefficient and mean characteristic path length, which were calculated based on 5000 randomly generated networks, all with the same degree distribution as the actually observed whole-brain network. Furthermore, we applied a data-driven subdivision of the network into modules (i.e. separated and non-overlapping sets of regions that are strongly connected to each other, and for this reason form independent modules), using the Louvain method (cfr. [65]). If single-region modules were obtained they were excluded from further analysis.

As for the nodal analysis, we computed the participation coefficient of each node[65] (See Supplementary Figure S2 for results relative to further nodal measures). The most

important nodes of the network, i.e. the hubs, were identified. Hubs were computed by selecting nodes whose participation coefficient was one standard deviation higher than the mean participation coefficient. The participation coefficient is an established index for hub identification and it has been proven to be more appropriate than other indices, as degree, at least for functional networks.[67]

Considering the sub-matrix based analysis, we calculated the total number of *within* or short-distance connections (i.e. connections linking two nodes in the same sub-matrix) and the total number of *between* or long-distance connections (i.e. connections linking two nodes belonging to different sub-matrices).

For the anatomically-driven networks, we computed the total number of connections and nodal degree *within* each network.

Statistical analysis

In order to rigorously test whether differences between DLB and HC were significant, we adopted a bootstrap procedure,[36] allowing hypothesis testing when the sample size is not so large to produce statistical inference. For each connectivity matrix, we extracted 5000 bootstrap samples of 42 subjects, with replacement, both for DLB and HC.

For each of the 5000 bootstrap samples, we calculated global and local metabolic connectivity indices and number of connections within and between sub-matrices or networks. We then performed multiple independent t-tests, using a Bonferroni correction. We tested global indices for each network and local indices for each node identified as a hub. We also compared the number of connections for each of the 10 sub-matrices in the whole-brain analysis, and for each network in the molecular network analyses.

Since the main results were consistent across matrices with different numbers of connections, we only reported the results relative to the most conservative threshold (i.e. 200 connections for the whole-brain analysis, 10 for α-synuclein analysis, 60 for dopamine systems analysis and 200 for cholinergic systems analysis).

Results

Whole-Brain Connectivity

Global measures – Segregation measures, i.e. clustering coefficient and modularity, were significantly decreased in DLB versus HC groups. As for integration measures, global efficiency increased, whereas characteristic path length decreased. All differences were statistically significant ($p<0.00001$). Small-worldness was higher than 1 for both DLB and HC.

Number of connections - DLB connectivity matrices showed a marked whole-brain metabolic connectivity reconfiguration, characterized by an increase of long-distance and a reduction of local connections (Figure 1; See Supplementary Table S5 for statistical details).

Modularity – The Louvain modularity method identified 6 modules for HC and 8 modules for DLB. In DLB, the nodes were sparsely relocated across modules with respect to HC, with extensive reconfigurations in frontal lobes, including the orbitofrontal cortex, in the parietal and occipital lobes, and in cerebellum (Figure 2A, for nodes and module reconfiguration).

Hubs – According to graph-theory, we identified 21 hubs for the HC group and 9 hubs for DLB. In DLB compared to HC, the hubs were classified into three categories, i.e.

lost, preserved and reconfigured hubs. The hubs changes was consistent with the whole network reorganization, described above. Notably, the lost and reconfigured hubs were part of frontal, parietal, occipital, thalamic and cerebellar regions (Figure 2B).

Sub-matrix Connectivity

Number of connections - As for the sub-matrix-based analysis, we found significant local connectivity decreases within a number of sub-matrices, namely in the occipital cortex, the cerebellum, thalamus and brainstem. There were in addition significant local connectivity increases within the parietal, temporal and frontal sub-matrices, as well as in the basal ganglia.

Regarding long-distance connections, we found significant functional disconnections in sub-matrices that also showed local alterations. Notably, in the frontal sub-matrix, we found decreased connectivity with occipital and cerebellum sub-matrices, but enhanced connectivity with parietal and basal ganglia sub-matrices; the thalamus presented widespread disconnections with occipital cortex, median cingulum, paracentral lobule, cerebellar and brainstem sub-matrices. The cerebellum showed selective decreases in connectivity with frontal, occipital, thalamic and brainstem sub-matrices. (see Figure 1B and Supplementary Table S6). All differences were statistically significant ($p<0.00001$).

Anatomically-driven analysis

Alpha-synuclein spreading

Connectivity analysis of α-synuclein Braak's stages showed altered connectivity in regions corresponding to Braak stages 1 to 4 (Figure 3). In particular, the projection from brainstem to hippocampal structures and amygdala were functionally disconnected.

The connectivity followed a specific gradient, characterized by a prevalent impairment in the brainstem regions, which following the Braak's hypothesis, represent the first regions to be affected. This result was consistent with the short disease duration of our sample.

Dopaminergic Networks

A severe loss of connections from dorsal striatum to the prefrontal, sensorimotor cortex, and the supplementary motor region characterized DLB. This network displayed highly interconnected nodes in HC. Differently, meso-limbic network connectivity remained more spared, with limited loss of connectivity from the ventral striatum (Figure 4). All differences were statistically significant ($p<0.00001$) (Supplementary Table S7).

Cholinergic Networks

A diffuse reconfiguration, characterized by both increased and decreased connectivity, was found in cholinergic networks (Figure 5). Specifically, Ch1-Ch2 and Ch5-Ch6 cholinergic networks showed decreases in the number of connections. Specifically, the Ch1-Ch2 network, hypothalamic regions were the most affected, together with the thalamic, midbrain and pontine regions within the Ch5-Ch6 network. In contrast, Ch3 and the lateral Ch4 perisylvian division networks presented with a global reconfiguration and connectivity increases between nodes. The medial and the lateral capsular Ch4 nuclei pathways showed a mixed profile, characterized by both increases and decreases in connectivity. Namely, the medial Ch4 showed a slight decrease in the number of connections to anterior and median cingulate regions, and an increase to the orbital regions and posterior cingulate gyrus. The lateral Ch4 capsular division showed decreased connectivity to the occipital lobe and, to a lesser extent, to the frontal superior medial regions, and a strong increase in the number of connections to the parietal lobe,

notably the angular gyri. All differences were statistically significant ($p<0.00001$) (Supplementary Table S8).

Discussion

A prevalent fraction of the brain glucose and energy metabolism is related to neuron activity,[12] particularly to neuronal communication, with up to 75% of signalling-related energy consumed post-syntaptically.[68] Even though, as reported by a recent *in vivo* study[69], neurons are the primary consumer of glucose, glia cells partially contribute to energy consumption, and may contribute to [18F]FDG-PET signal. However, with an estimated 25% of the total brain energy use being dedicated to housekeeping function, [18F]FDG-PET signal primarily reflects energy consumption related to: 15% resting potentials, 16% action potentials and 44% to synaptic processes.[95] Thus, [18F]FDG-PET might be considered an ideal surrogate for energy consumption in neurons, and specifically in synapses[10–12,69], also representing a unique *in vivo* tool for capturing the heterogeneous events that contribute to synaptic dysfunction and consequent neurodegeneration.[70] Notably, the role of [18F]FDG-PET in tracing neuronal dysfunction and supporting early dementia diagnosis is widely recognized.[9,29,30,71]

Novel insights about the possible presence of widespread neurodegeneration effects can be provided by the analysis of complex metabolic connectivity brain networks. This is the first study assessing brain metabolic connectivity in DLB, using graph theory and SICE method. Previous reports investigated functional connectivity in DLB by using fMRI, applying seed based and independent component analysis (ICA) methods.[23–28]

Seed-based analysis relies on an *a priori* selection of brain regions, that are used as seeds to perform correlation analysis.[23,24] For this reason, seed-based studies might suffer by the choice of an *a priori* limited number of seed regions, preventing a more comprehensive assessment of functional connectivity. The ICA, on the other hand, allows the assessment of whole-brain resting state networks, which were found altered in DLB.[27,28] All in all, the fMRI studies have provided mixed results, possibly related to the relatively small sample size (ranging from a minimum of 15 to a maximum of 18 subjects), to the variable disease duration, to the clinical heterogeneity characterizing the sample, and to the different techniques used to assess functional connectivity (seed-based versus independent component analysis). For example, one of the less consistent finding reported in fMRI connectivity studies in DLB, concerns the altered connectivity in the occipital cortex. For example, a seed-based study has shown decreased functional connectivity between visual cortex and precuneus,[23] whereas Kenny and colleagues (2012) reported no differences in connectivity between primary visual cortex and other regions,[24] that, however, emerged when using the ICA approach.[27]

Using SICE, we exploited the unique ability of [18F]FDG-PET to *in vivo* investigate resting state cerebral metabolism, in order to obtain metabolic connectivity and complex brain network indices based on graph theory.[72] The results of the multivariate analyses, as SICE, represent a new assessment brain metabolism dysfunction based on altered connectivity, which is not dependent nor predicted by results obtained with univariate analyses. Moreover, our whole brain connectivity analyses were performed using a standardized anatomical atlas, i.e. AAL, which assures feature selection independent from hypo- and hypermetabolic patterns.

Following an innovative metabolic and molecular perspective, we investigated the metabolic connectivity in the whole brain and in anatomically-driven networks on the basis of DLB neuropathology, namely, the proposed progressive spreading of α-synuclein pathology,[6,7] and according to the dopaminergic and cholinergic neurotransmission alterations.

Whole-brain analysis

The analysis of whole-brain metabolic connectivity showed a widespread reconfiguration of brain functional architecture in DLB. Namely, graph-theory measures revealed altered connectivity network properties, characterized by a reduction of small-scale and an increase of large-scale connectivity. The resulting imbalance, characterized by decreased segregation and increased integration, reflects a modular dissociation and a consequent functional disorganization of the brain connectome in DLB (Figure 1A). The decreased network modularity suggests the presence of functionally less specialized communities of nodes (Figure 2A).[65] Connectivity disorganization is also consistent with hub loss, since hubs play a key role in the global organization and efficiency of the brain connectome.[65] In spite of this global reconfiguration, the small-world network organization remained preserved, as previously found in other neurodegenerative diseases.[63,73] The small-worldness however, is a synthetic index and it may falsely report local integrity, particularly when one of the two reported measures (i.e. segregation and integration), is high enough to compensate for the impairment of the other.[65]

Brain local connectivity

<u>*Decreases*</u> - A severe disruption of local connectivity was found in the occipital cortex. Occipital lobe dysfunction is associated to simple hallucinations[74] and to the visuo-perceptual deficits observed in DLB.[75]

Cerebellar, thalamic, and brainstem sub-matrices were also affected. Cerebellum is involved in the coordination and temporal organization of various motor and non-motor (e.g. executive and attentional) processes.[76] Recent findings suggest that it may contribute to some neuropsychological features of DLB, such as executive and working memory dysfunctions.[77] Thalamus has a central role in visual perception, attention, and alertness,[78,79] functional domains impaired in DLB.[1] The loss of local connectivity in these structures is compatible with recent models of visual hallucinations, described as the consequence of the combined impairment of both visuo-perceptive and attentional systems.[74] Local connectivity was also reduced in the brainstem, a region widely affected by α-synuclein pathology. Neuronal losses in its neurotransmitters nuclei were reported in pathological studies of DLB,[15,16] and related to the presence of RBD.[80] Moreover, the integrity of brainstem connectivity, in particular of ascending reticular activating system nuclei, is critical for arousal maintenance,[78] which shows fluctuations in DLB.[1]

Increases - Frontal, temporal, parietal and striatal sub-matrices displayed increased local connectivity. A local reconfiguration, with residual nodes replacing functionally impaired nodes, was found in these regions. This increase in connectivity was reported in other dementia conditions, and was related to compensatory mechanisms still possible in early disease phase, such as over-recruitment of partially spared neural function.[22]

Long-range connectivity

Decreases - The analysis of long-range connectivity revealed functional disconnections between occipital, frontal, and cerebellar sub-matrices. Fronto-occipital metabolic and structural abnormalities were previously reported in DLB, and related to depressive

mood and visual hallucinations.[81,82] Cerebello-occipital disconnections might explain DLB visual-attentional impairment, given the role of the cerebellum in modulating visual areas activity under attentional demands.[83] The here reported cerebello-frontal disconnection could be part of the cerebello-thalamo-cortical circuit, in which contributes to executive, attentional, and working memory processes.[76] Cerebellar metabolic connectivity was affected at both a small and large-scale level, in accordance to the reported widespread functional, anatomical, and pathological alterations in DLB.[77,84,85] Further studies are necessary to fully disclose the role of the cerebellum in DLB.

Widespread long-range disconnections were crucially found between the thalamus, the superior parietal, occipital, and cingulate cortices. The thalamus is indeed a crucial relé in circuits that are relevant for the motor, cognitive, and behavioural symptoms.[86] Considering the α-syncleopathies, recent fMRI resting state studies reported the involvement of striato-thalamo-cortical in Parkinson's disease.[87]

Increases - The same sub-matrices showing local increases of connectivity, also displayed enhanced long-range connectivity, suggesting a more complex reorganization, associated with disruption of the functional hierarchy characterizing the normal brain (Figure 1).

Anatomically-driven networks

As a main contribution of the present study, we adopted a new prospective to analyse selective vulnerability of metabolic connectivity networks, by targeting well-known molecular and neurotransmission alterations in DLB neuropathology.

The connectivity analysis within networks affected by α-synuclein pathology showed alterations in regions corresponding to α-synuclein Braak stages 1-4, with a greater

impairment of Braak stages 1-2, consistent with the short disease duration characterizing our sample (mean±SD=2.14±1.4 years) (Figure 3). In particular, medulla oblongata (stage 1), pons (stage 2) and midbrain (stage 3) were characterized by the greatest metabolic connectivity impairment, followed by the impairment in the connected limbic structures (i.e. hippocampal structures, ventral striatum, amygdala), confirming the causal relationship between α-synuclein accumulation and synaptic dysfunction. Our connectomics analysis supports Braak's staging for the spreading of α-synuclein neuropathology in DLB.[6,7]

The assessment of metabolic connectivity within the dopaminergic networks showed a prevalent impairment of the striato-cortical pathway (Figure 4). The striato-cortical pathway involvement is a hallmark in DLB disease processes, and the substantia nigra is one of the main sites of α-synuclein aggregation.[8] Alpha-synuclein accumulation interferes with neurotransmitter release at presynaptic sites, producing widespread effects on dopaminergic projections.[8] The meso-limbic pathway, assessed here for connectivity integrity, showed preserved functional configuration, consistently with the reported resistance of ventrotegmental area neurons to α-synuclein pathology.[88]

The cholinergic networks also underwent important reconfigurations at different levels (Figure 5). The Ch1-Ch2 and Ch5-Ch6 pathways had the most important reduction in connectivity, possibly suggesting an involvement of these nuclei as an early pathogenic event in DLB. Previous anatomo-pathological studies have shown neuronal loss in these regions,[15,89] and cholinergic impairment was reported *in vivo* in the subcortical regions they supply.[18]

The cholinergic system has been implicated as potential mechanism in the pathophysiology of visual hallucinations and cognitive fluctuations characterizing

DLB.[90] The impairment of Ch5-Ch6 pathway here reported, supports its involvement in the pathophysiology of DLB. In particular, the prevalent brainstem-thalamic connectivity impairment is consistent with an involvement of peduncolopontine and thalamic regions in the genesis of visual hallucinations.[91]

Furthermore, the brainstem-thalamic connectivity derangement may be also a fundamental basis in the clinical manifestation of cognitive fluctuations.[79]

Moreover, the involvement of Ch4 projections from the nucleus basalis of Meynert to cortical structures, in particular to the frontal and occipital regions, is consistent with previous reports.[17,18,92] There is evidence of neuronal loss in nucleus basalis of Meynert and reduction of AchE activity in the neocortical regions supplied by this nucleus, as measured by [11C]-MP4A PET.[14,18,93] The presence of α-synuclein pathology in nucleus basalis of Meynert, in addition to neurodegeneration processes, may contribute to reduction of neocortical cholinergic activity in DLB.[92]

All in all, we assume that the big part of the [18F]FDG-PET metabolic connectivity alterations we found are mainly due to the neuropathological events affecting synaptic signalling in DLB, such as aggregates of α-synuclein at presynapses,[8] neuroinflammatory process,[96] and neurotransmitters alterations.[11]

This study has some possible limitations and strengths**.** The anatomically-driven analyses were performed using anatomical regions not specifically constructed for the targeted brain systems. In the future, atlases based on neurotransmitter mapping and/or histopathology may become available, allowing this limitation to be overcome.

It must be acknowledged that *in vivo* [18F]FDG-PET imaging, although allowing the investigation of molecular and metabolic mechanisms at nanomolar levels, has the limit of spatial resolution. [18F]FDG-PET imaging is a unique tool for *in vivo* tracking of

pathology in humans and future studies combining other microscopic and molecular methods, may allow establishing more direct links between metabolic alterations and the underlying cellular and synaptic pathology.

Conclusions

Our work explored for the first time the DLB global metabolic connectivity endophenotype, in relation to the histopathology and specific neurotransmission pathways. These results offer new insights to the clinical phenotypes of DLB, useful for future clinico-functional correlation studies. This work focused on DLB at an early disease phase, showing the specific early vulnerability of different networks on a metabolic basis. These results allow for a more comprehensive neuro-functional picture in DLB, paving the way for new research perspectives. For example, assessing the global and local changes in whole-brain functional and molecular networks at later disease phases that could enhance the understanding of DLB progression; the relationship between DLB altered connectivity endophenotype and other synucleinopathies might be a future issue of researches, since diseases sharing the same molecular pathology might present similar vulnerabilities.[2,21] Finally, the future integration of metabolic and functional measures, as provided by combined [18F]FDG-PET and fMRI methods,[35] might provide essential information on the directionality of connections, thus adding knowledge on the altered signalling hierarchies in DLB.

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Author Contributions

S.P.C., M.T. and A.S. performed the analyses and wrote the manuscript. L.P. processed data, S.I. and G.M. performed clinical assessments and provided clinical diagnosis. S.F.C. wrote the manuscript. D.P. designed research and wrote the manuscript. All authors reviewed the manuscript.

Disclosure/Conflict of interest

The authors declare no competing financial interests.

Supplementary Information

Supplementary material for this paper can be found at http://jcbfm.sagepub.com/content/by/supplemental-data**.**

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Figure Legends

Figure 1. Whole-brain metabolic connectome in DLB and HC.

(**A**) The first column displays correlation matrices of DLB and HC obtained from the analysis of the full-matrix composed by 121 nodes. The red-yellow colour gradient represents the weight of the correlation between nodes, whereas coloured squares indicate the nodes' anatomical localization. The second column displays DLB and HC brain connectivity graphs on a 3D brain template. Only the strongest connections are presented (in yellow). The dimension of each node depends on the node total number of connections, whereas the colour indicates its anatomical localization. A global connectivity derangement and reconfiguration is evident in DLB versus HC, with the pathological group showing a loss of the hierarchical functional skeleton found in healthy subjects. (**B**) The T-score matrix shows differences in the number of connections within and between each sub-matrix in DLB versus HC. Abbreviations: F: Frontal; PCL: Paracentral Lobule; MCC: Median Cingulate Cortex; ROL: Rolandic

Operculum; P: Parietal; O: Occipital; T: Temporal; In: Insula; Th: Thalamus; BG: Basal Ganglia; BS: Brainstem; Cbl: Cerebellum.

Figure 2. Modules and hubs.

(**a**) Modular subdivision of DLB and HC whole-brain networks, performed with Louvain modularity method. An overall poorly delineated modular organization is present in DLB. (**b**) Hub reconfiguration in DLB. Blue circles represent lost hubs (hubs present in HC but not in DLB); green circles indicate preserved hubs (hubs present in both DLB and HC); red circles show reconfigured hubs (nodes that in DLB assume the role of hubs differently to HC).

Figure 3. Alpha-synuclein network analysis.

Regions affected by α-synuclein at stages 1 to 4. Greater impairment is found in regions corresponding to the earlier stages (i.e. 1-2), consistently with the short disease duration characterizing our sample.

Figure 4. Dopaminergic networks analysis.

Cortical and subcortical projections of the two main dopaminergic pathways. The prevalent impairment of the striato-cortical pathway is clearly visible, with a huge disruption of functional connections arising from the dorsal striatum. The meso-limbic pathway is relatively spared, even if a general reconfiguration is found.

Figure 5. Cholinergic networks analysis.

Cortical and subcortical projections of the six main cholinergic pathways. The first row shows regions supplied by Ch1-Ch2 and Ch3 basal forebrain and Ch5-Ch6 brainstem nuclei. The second row shows projections of the three pathways innervating from the

nucleus basalis of Meynert (Ch4). Loss of connectivity is observed in regions supplied by Ch1-Ch2 and Ch5-Ch6 nuclei. Ch3 and Ch4 projections displayed variable reconfigurations with altered connectivity profiles.