UNIVERSITA' VITA-SALUTE SAN RAFFAELE

CORSO DI DOTTORATO DI RICERCA
IN NEUROSCIENZE COGNITIVE

Sleep timely transitional markers for synucleinopathies

DoS: Dr. Andrea Galbiati

Tesi di DOTTORATO di RICERCA di

Giada D'Este

matr. 019549

Ciclo di dottorato XXXVII°

SSD M-PSI/02

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This thesis has been:

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- has been written according to the editing guidelines approved by the University.

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All the results presented here were obtained by myself, except for:

1) ***[^{18}F]FDG–PET analyses and SW-SO detection (Study II, chapter 3):***

- *[^{18}F]FDG–PET image pre-processing and iRBD subject expression of PDRP were performed by Dr Giulia Carli (Neurology Department, University of Michigan, Ann Arbor, MI, USA).*

- *SW and SO detection using YASA algorithm was performed by Dr Caterina Leitner (Department of Psychology, "Vita-Salute" San Raffaele University, Milan, Italy; Sleep Disorders Centre, Department of Clinical Neurosciences, Neurology, IRCCS San Raffaele Scientific Institute, Milan, Italy).*

All sources of information are acknowledged by means of reference.

I would like to acknowledge that the first study included in the third chapter of the present dissertation (Study I, chapter 3) has been previously published in D'Este, G., Berra, F., Carli, G., Leitner, C., Marelli, S., Zucconi, M., Casoni, F., Ferini-Strambi, L. & Galbiati, A. (2023). Cognitive Reserve in Isolated Rapid Eye-Movement Sleep Behavior Disorder. Brain Sciences, 13(2), 176. DOI: https://doi.org/10.3390/brainsci13020176.

Dedication

In a hole in the ground there lived the fellowship of the Sleep Disorders Center.

Here is where my adventure begins. My PhD journey has been shaped by my burning passion for research, but also by self-sacrifice. The result of this long journey is the present thesis (my precious). However, this wasn't a solo adventure. I shared this path with a huge, unexpected party. In the following pages I want to thank all the players that joined it over the years.

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Well, here at last, dear colleagues and friends, comes the end of this unexpected journey. I will not say: do not weep, as a wise wizard once stated, not all tears are evil.

Enjoy the thesis!

Abstract

Isolated rapid eye-movement (REM) sleep behavior disorder (iRBD) is classified as a REM sleep parasomnia, that takes its stand at the beginning of the α-synucleinopathies spectrum. Since its role as an important risk factor of conversion within this field, the identification of timely biomarkers, but also protective factors against neurodegeneration is a leading objective. By means of three different studies, my PhD dissertation aimed to explore the role of behavioral, neuropsychological and neurophysiological measures, as possible early markers for disease progression in iRBD patients. The first study explored the role of cognitive reserve (CR) in iRBD in two ways. First, we evaluated the impact of CR on the cognitive functioning of iRBD patients and provided the first preliminary findings supporting CR as a possible protective factor in iRBD. Second, we combined CR and brain reserve (BR) measures in order to evaluate their interaction and influence on pathological burden and cognitive performance in iRBD. In this context, we not only replicated previous evidence linking slow waves (SW) disruption to cognitive impairment, but we also suggested a novel interaction between pathological burden and SW sleep dynamics in iRBD. The second study aimed to explore the possible presence of early peripheric cholinergic dysfunction in iRBD. The results demonstrated the presence of pupillary light reflex alterations which were associated with poorer performances in visuospatial and visuoperceptive processing, further indicating the presence of an underlying cholinergic dysfunction and supporting the role of pupillometry in this context. Finally, the third was a pilot study with the aim of evaluating the possible presence of temporal processing and sensorimotor synchronization dysfunctions in iRBD and to investigate resting-state electroencephalographic rhythms. The results suggested the presence of an executive-based dysfunction underlying timing-related impairments in iRBD and provided further insights into the nature of individual's intrinsic rhythmicity.

Altogether these different aspects of iRBD pathology shaped a complex puzzle concerning the different dysfunctions, pathophysiological causes, disease mechanisms and future clinical trajectories that these patients may come across in their path. The present PhD dissertation adds a little piece to the puzzle of the current knowledge in this field, but I hope that this will contribute to paving the way for future therapeutic research.

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ACRONYMS AND ABBREVIATIONS

[18F]FDG	18fluorodeoxyglucose
[18F]FDG-PET	18fluorodeoxyglucose positron emission tomography
123I-FP-CIT	Iodine 123-radiolabeled 2β-carbomethoxy-3β-(4-iodophenyl)-N-(3-fluoropropyl) nortropane
123I-MIBG	123iodine-metaiodobenzylguanidine
123I-MIBG-SPECT	123iodine-metaiodobenzylguanidine single photon emission-computed tomography
AASM	American Academy of Sleep Medicine
ACh	Acetylcholine
ACmax	Maximum constriction acceleration
AD	Alzheimer's disease
ADMN	Anterior default mode network
AIC	Akaike information criterion
ALP	Autophagy-lysosome pathway
aMCI	Amnestic mild cognitive impairment
aMCImd	Amnestic mild cognitive impairment multi-domain
aMCIsd	Amnestic mild cognitive impairment single-domain
ANS	Autonomic nervous system
APOE	Apolipoprotein E
ASR	Age-standardized rate
AUC	Area under the curve
Aβ	Amyloid-β
BAASTA	Battery for the assessment of auditory sensorimotor and timing abilities
BAND Lab	Basic & Applied NeuroDynamics Laboratory
BIN1	Bridging integrator 1

Bpm	Beats per minute
BR	Brain reserve
CA	Cluster analysis
Ca2+	Calcium
CIS	Cingulate island sign
CN	Cranial nerve
CNS	Central nervous system
CR	Cognitive reserve
CRIq	Cognitive reserve index questionnaire
CSF	Cerebrospinal fluid
CT	Computed tomography
CV	Coefficient of variation
d'	d-prime
DA	Dopamine
DAT	Dopamine transporter
DAT-SPECT	Dopamine transporter single photon emission-computed tomography
DJ-1	Protein deglycase DJ-1 (or Parkinson's disease protein 7)
DLB	Dementia with Lewy bodies
DNAJC6	DnaJ heat shock protein family 40 member C6
EANM	European Association of Nuclear Medicine
ECG	Electrocardiography
EEG	Electroencephalography
EMG	Electromyography
ENS	Enteric nervous system
EOG	Electrooculography
FA	False alarm
FFT	Fast Fourier transform

FoG	Freezing of gait
fps	Frames per second
GBA	Glucocerebrosidase
GBD	Global Burden for Disease
GP	Globus pallidus
Gpi	Globus pallidus internus
GWAS	Genome-wide association study
H/M	Heart-to-mediastinum
IADL	Instrumental activities of daily living
IAPF	Individual resting alpha peak frequency
ICA	Independent component analysis
ICSD	International Classification of Sleep Disorders
INPP5F	Inositol polyphosphate-5-phosphatase F
IOI	Inter-onset-interval
IQR	Interquartile range
iRBD	Isolated rapid eye-movement sleep behavior disorder
iRBD+MCI	Isolated rapid eye-movement sleep behavior disorder with mild cognitive impairment
iRBD-MCI	Isolated rapid eye-movement sleep behavior disorder without mild cognitive impairment
ISI	Inter-stimulus-interval
ITI	Inter-tap-interval
K+	Potassium
KC	K-complex
KCd	K-complex density
LAG3	Lymphocyte-activation gene 3
LBD	Lewy bodies disorders
LBs	Lewy bodies

L-DOPA	Levodopa
LNs	Lewy neurites
LPT	Lateral pontine tegmentum
LRRK2	Leucine-rich repeat kinase 2
M1 microglia	Pro-inflammatory reactive microglia phenotype
MAPT	Microtubule-associated protein Tau
MCI	Mild cognitive impairment
MCImd	Mild cognitive impairment multi-domain
MCIsd	Mild cognitive impairment single-domain
MCRF	Magnocellular reticular formation
MDS	Movement Disorder Society
ML	Maximum likelihood
mm	Millimeters
mm/s	Millimeters per second
MMSE	Mini-Mental state examination
MNI	Montreal neurological institute
MRI	Magnetic resonance imaging
ms	Milliseconds
MSA	Multiple system atrophy
N1	Stage 1 of non-rapid eye-movement sleep
N2	Stage 2 of non-rapid eye-movement sleep
N3	Stage 3 of non-rapid eye-movement sleep
NA	Noradrenaline
Na+	Sodium
naMCI	Non-amnestic mild cognitive impairment
naMCImd	Non-amnestic mild cognitive impairment multi-domain
naMCIsd	Non-amnestic mild cognitive impairment single-domain

NIA	National institute on aging
NKA	Na+/K+-ATPase
NMSQ	Non-motor symptoms questionnaire
NREM	non-rapid eye-movement
OH	Orthostatic hypotension
OSA	Ostructive sleep apnea
PANS	Parasympathetic nervous system
PC	Pre-coeruleus
PD	Parkinson's disease
PD+MCI	Parkinson's disease with mild cognitive impairment
PDD	Parkinson's disease dementia
PD-MCI	Parkinson's disease patients without mild cognitive impairment
PDRP	Parkinson's disease-related pattern
PET	Positron emission tomography
PFC	Prefrontal cortex
PICA	Probabilistic independent component analysis
PIGD	Postural instability and gait difficulties
PINK1	Phosphatase and tensin homolog-induced kinase 1
PK	Proteinase K
PLM	Periodic limb movement
PLR	Pupillary light reflex
PNS	Peripheric nervous system
PPT	Preferred perceptual tempo
Prion	PRoteinaceous Infective ONly particle
PRKN	Parkin
PrPc	Prion Protein Cellular
PrPSc	Prion Protein Scarpie

PSG	Polysomnography
RAVLT	Rey auditory verbal learning test
RBD	Rapid eye-movement sleep behavior disorder
RBDRP	Rapid eye-movement sleep behavior disorder-related pattern
REM	Rapid eye-movement
ROCF	Rey–Osterrieth complex figure
ROI	Region of interest
RPM	Raven's progressive matrices test
RSWA	Rapid eye-movement sleep without atonia
SANS	Sympathetic nervous system
SCARB2	Scavenger receptor class B member 2
SCFA	Short chain fatty acids and vitamins
SCOPA-AUT	Scale for outcomes in Parkinson's disease – autonomic
SLD	Sublaterodorsal nucleus
SMA	Supplementary motor area
SMT	Spontaneous motor tapping
SN	Substantia nigra
SN	Substantia nigra
SNAREs	Soluble N-ethylmaleimide-sensitive factor attachment protein receptors
SNCA	Synuclein α
SNpc	Substantia nigra pars compacta
SO	Slow oscillations
SOd	Slow oscillations density
SPECT	Single photon emission-computed tomography
SPSS	Statistical package for social sciences
sRBD	Secondary/symptomatic rapid eye-movement sleep behavior disorder

SS	Sleep spindles
SSM	Scaled subprofile model
SSM/PCA	Scaled subprofile model/principal component analysis
SW	Slow waves
SWA	Slow waves activity
SWd	Slow waves density
syn	Synuclein
TH	Tyrosine hydroxylase
TMEM175	Transmembrane protein 175
TMT	Trial making test
TNTs	Tunneling nanotubes
t-SNAREs	Target-membrane SNAREs
UMCG	University Medical Centre Groningen
UPDRS	Unified Parkinson's disease rating scale
UPP	Ubiquitin–proteasome pathway
VCmax	Maximum constriction velocity
VGCCs	Voltage-gated Ca2+ channels
vlPAG	Ventrolateral part of the periaqueductal gray matter
VMAT2	Vesicular monoamine transporter 2
VOSP	Visual object and space perception
VPS13C	Vacuolar protein sorting 13 homolog C
VPS35	Vacuolar protein sorting ortholog 35
v-SNAREs	Vesicle membrane SNAREs
WHO	World Health Organization
WR	Washout rate
YASA	Yet another spindle algorithm
α-syn	α-synuclein

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1. INTRODUCTION

1.1. The Alpha-synucleinopathies Spectrum

The α-synucleinopathies spectrum is an umbrella-term which refers to a range of neurodegenerative disorders with different clinical features, but all characterized by the pathological accumulation of the protein α-synuclein (α-syn) which leads to the formation of the characteristic inclusions of these disorders i.e., Lewy bodies (LBs) and Lewy neurites (LNs) (Calabresi et al., 2023; Calo et al., 2016; Goedert, 2001). This spectrum can be considered as evolving through different stages, from the prodromal isolated rapid eye-movement (REM) sleep behavior disorder (iRBD) and extending through overt neurogenerative disorders, mainly Parkinson's disease (PD), PD dementia (PDD) and dementia with Lewy bodies (DLB) (Calabresi et al., 2023). This introductory chapter will provide a comprehensive overview of the α-synucleinopathies spectrum, starting from its neurobiological bases and continuing by describing the main conditions that fall under this category, each characterized by varying degrees of severity and different clinical features.

1.1.1. Alpha-synuclein: from function to pathology

Synucleins are defined as a family of proteins widely expressed in the brain. Specifically, they were discovered by Maroteaux and collaborators in 1988 (Maroteaux et al., 1988) in the presynaptic nerve terminals of the Torpedo electric ray, hence their name (Bougea, 2021; Goedert, 2001). This family of small, natively unfolded, soluble proteins includes three pillars: α-syn, β-syn and γ-syn (Goedert, 2001). The structure of human α-syn is 140 amino acid–long divided into (Bougea, 2021; Calo et al., 2016; Goedert, 2001; Vekrellis et al., 2004) (see Figure 1):

1. Amphipathic N-terminal domain (amino acids 1–60) with an apolipoprotein lipid-binding motif. Of note, the N-terminal domain contains seven imperfect repeats of 11 amino acids, each with the six-consensus sequence KTKEGV.
2. NAC central hydrophobic core (amino acids 61–95) which contains the non-amyloid-β (Aβ) component region involved in protein aggregation and fibril formation.
3. Acidic C-terminal domain (amino acids 96–140) involved in aggregation

suppression.

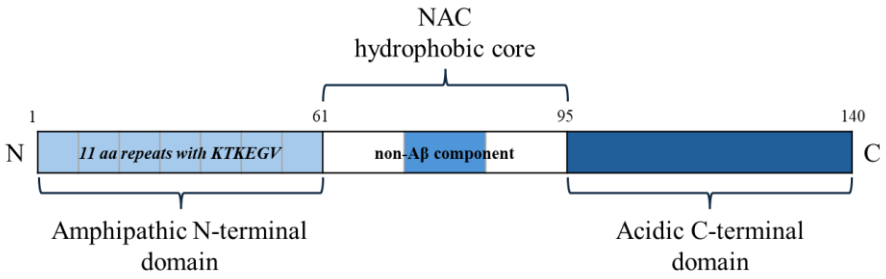


Figure 1. Schematic representation of α-syn structure. This figure was created by me based on Vekrellis et al, 2004. Abbreviations: aa, amino acids; Aβ, amyloid-β.

In humans, α-syn is physiologically localized in presynaptic terminals as a natively unfolded monomer in the cytoplasm or with an α-helical multimeric conformation when anchored to synaptic vesicles (Bougea, 2021; Calo et al., 2016). Of note, while this helical form appears to be fundamental to the physiological functions of α-syn and it seems to protect against aggregation, the unfolded form seems to be strongly involved in pathological aggregation (Calo et al., 2016; Mavroeidi & Xilouri, 2021). Regarding the physiological roles of α-syn, they can be summarized into four main functions: a) regulation of fusion and clustering of vesicles; b) modulation of neurotransmitter release (i.e., dopamine (DA)); c) modulation of synaptic plasticity; d) involvement in other basic cellular functions (Bougea, 2021; Calo et al., 2016; Mavroeidi & Xilouri, 2021; Vekrellis et al., 2004). In the context of fusion and clustering of vesicles, α-syn acts as a significant contributor in the formation of the "SNARE complex" (see Figure 2). SNAREs (Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptors) are a family of fusion proteins involved in mediating the fusion of vesicles with target membranes in various cellular processes and, based on their location and function, they can assume two forms: vesicle membrane SNAREs (v-SNAREs) and target-membrane SNAREs (t-SNAREs) (Kandel et al., 2013). The binding of α-syn to the synaptic plasma membrane ensures the alignment and interaction of SNAREs proteins and, by keeping v-SNAREs and t-SNAREs together, it leads to the formation of the SNARE complex allowing vesicle docking and fusion to take place (Calo et al., 2016; Kandel et al., 2013).

This is essential for the second function of α-syn: the neurotransmitter release (see Figure 2). Indeed, it has been demonstrated that α-syn acts as a key regulator of DA

homeostasis by being involved in synaptic vesicle recycling, DA storage, DA release, DA transporter activity, DA reuptake and DA Biosynthesis (Calo et al., 2016; Yu et al., 2005). In this context, α-syn influences DA storage and release not only by regulating the dimension and secretion properties of both synaptic vesicles recycle and reserve pools during the synaptic vesicle cycle; but also, by directly interacting with the vesicular monoamine transporter 2 (VMAT2) which plays a crucial role in loading neurotransmitters into synaptic vesicles (Calo et al., 2016; Yu et al., 2005). Indeed, α-syn also plays a key role during DA reuptake from the synaptic cleft back into presynaptic neurons by downregulating the activity of the DA transporter (DAT) (Butler et al., 2015; Yu et al., 2005). Finally, α-syn also regulates DA biosynthesis by influencing the activity of the enzyme tyrosine hydroxylase (TH), which converts tyrosine into levodopa (L-DOPA) which is subsequently transformed into DA (Mavroeidi & Xilouri, 2021; Yu et al., 2005).

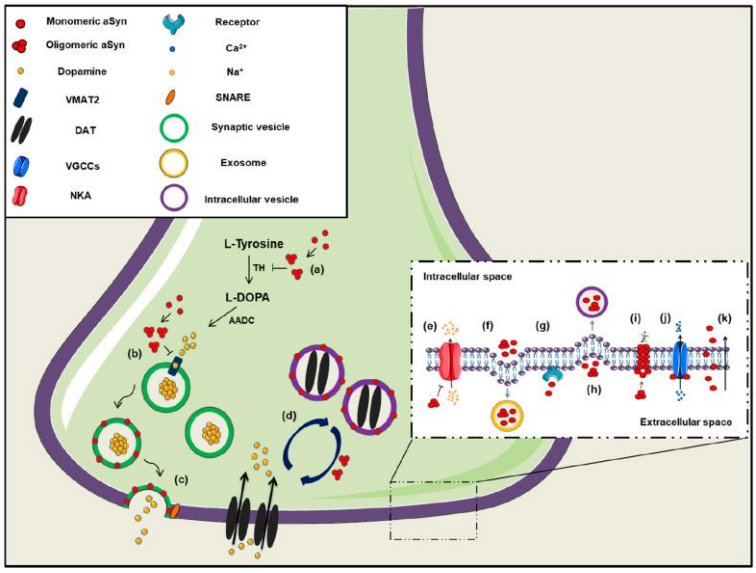


Figure 2. Schematic representation of α-syn roles: from function to pathology. This figure was reprinted from Mavroeidi & Xilouri, 2021 (Open Access Source). (a) Dopamine (DA) biosynthesis: α-syn effects on TH. (b) DA uptake: α-syn effects on VMAT2. (c) Fusion of vesicles and neurotransmitter release: α-syn effects on SNARE-dependent vesicle fusion and DA release. (d) DA re-uptake: α-syn effects on DA transporter. (e-f) extracellular α-syn effects on plasma membrane, from its secretion to passive diffusion/direct penetration into neuronal cells. Abbreviations: Ca^{2+}, Calcium; DAT, dopamine transporter; K^{+}, potassium; L-DOPA, levodopa; Na^{+}, sodium; NKA, Na^{+}/K^{+}-ATPase; SNARE, Soluble N-ethylmaleimide-sensitive Factor Attachment Protein Receptors; TH, tyrosine hydroxylase; VGCCs, voltage-gated Ca^{2+} channels; VMAT2, vesicular monoamine transporter 2; αSyn, α-synuclein.

The role played by α-syn in the regulation of the synaptic vesicle cycle and, consequently, in neurotransmitter release seems to be strictly related to its role in synaptic plasticity (Calo et al., 2016; Cheng et al., 2011; Di Rosa et al., 2003). Indeed, the localization of α-syn in presynaptic nerve terminals of brain regions characterized by synaptic plasticity leaded to explore its short- and long-term effects (Cheng et al., 2011). Concerning long-term synaptic plasticity, different studies demonstrated that α-syn is involved in the mechanisms underlying both long-term potentiation and depression by influencing the signaling pathways that mediate changes in synaptic strength (Cheng et al., 2011; Gureviciene et al., 2007, 2009; Liu et al., 2004, 2007; Watson et al., 2009). This evidence supports the potential impact of α-syn on learning and memory functioning (Cheng et al., 2011).

Finally, several studies reported the involvement of α-syn in other basic cellular functions which are strictly linked to its aggregation and degradation i.e., the autophagy-lysosome pathway (ALP) and the ubiquitin–proteasome pathway (UPP) (Betarbet et al., 2005; Calo et al., 2016; Chung et al., 2001; Ebrahimi-Fakhari et al., 2011; Ross & Pickart, 2004; Sahoo et al., 2022). Both ALP and UPP can be defined as cellular mechanisms essential for the degradation and recycling of waste products, among which misfolded proteins (Sahoo et al., 2022). For this reason, pathological changes in α-syn aggregation not only have a profound effect on ALP and UPP pathways, but the alteration of these pathways heightens α-syn pathology within a dual relationship (Sahoo et al., 2022).

All these data corroborate the role of α-syn in a broad-spectrum of mechanisms, thus reflecting its conformational flexibility (Lashuel et al., 2013). As previously mentioned, α-syn is physiologically localized in presynaptic terminals as a natively unfolded monomer in the cytoplasm or with an α-helical multimeric conformation when anchored to synaptic vesicles (Calabresi et al., 2023; Calo et al., 2016; Goedert, 2001). However, building upon these forms, α-syn is capable of aggregating into a variety of structural configurations which may lead to synaptic dysfunction and subsequent neural death (Calo et al., 2016; Cascella et al., 2022; Heras-Garvin & Stefanova, 2020; Ingelsson, 2016; Lashuel et al., 2013) (see Figure 3). In the cytoplasm, two unfolded monomers of α-syn may merge into an unstable (propagating) dimer and, by proliferating, form spherical and ring-like oligomers (Lashuel et al., 2013; Steiner et al., 2011). Further aggregation of α-syn to these structures may lead to the creation of amyloid fibrils, then resulting in the

formation of the characteristics LBs and LNs (Lashuel et al., 2013; Steiner et al., 2011). On the other hand, when anchored to synaptic vesicles, α-syn assumes an α-helical multimeric conformation (Calo et al., 2016; Goedert, 2001). Further aggregation of α-syn to this structure may contribute to the creation of membrane-bound β-sheet-rich intermediates, eventually resulting in the assembly of oligomers and fibrils (Lashuel et al., 2013; Steiner et al., 2011). Of note, some oligomers will form fibrils and, lastly, LBs/LNs (on-fibrillar assembly pathway), meanwhile others will preserve the oligomeric configuration (off-fibrillar assembly pathway) (Alam et al., 2019; Cascella et al., 2022; Ingelsson, 2016). In this context, several studies demonstrated that oligomers and fibrils exhibit greater neurotoxic properties compared to LBs and LNs (Alam et al., 2019; Cascella et al., 2022; Ingelsson, 2016; Lashuel et al., 2013; Ono, 2017; Verma et al., 2015). Particularly, oligomers seem to be even more toxic than fibrils per se due to the structural and functional properties of the variety of intermediate oligomers (Cascella et al., 2022; Ingelsson, 2016).

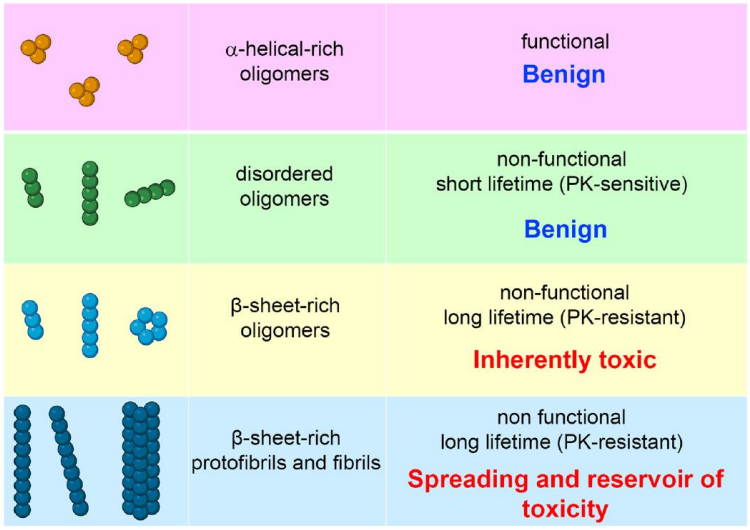


Figure 3. Schematic representation of the main α-syn configurations and their properties. This figure was reprinted from Cascella et al., 2022 (Open Access Source). Abbreviations: PK, proteinase K.

Among the main neurotoxic effects of these pathological α-syn structures, several studies demonstrated that oligomers are capable of perturbing cellular homeostasis by forming pores within the plasma membrane, thus amplifying membrane permeabilization and, consequently, the influx of calcium ions from the extracellular space (Ingelsson,

2016; Lashuel et al., 2013; Ono, 2017). Moreover, given the role of α-syn in SNARE complex formation, the accumulation of oligomers may disrupt the synaptic vesicle cycle causing synaptotoxic effects (Ingelsson, 2016; Lashuel et al., 2013; Ono, 2017). As briefly mentioned, the dual relationship between ALP/UPP pathways and α-syn may also contribute to exacerbate α-syn pathology. Indeed, deficits in the clearance of pathological α-syn structures may cause increased protein aggregation leading to profound neurotoxic effects (Alam et al., 2019; Ingelsson, 2016; Sahoo et al., 2022). Finally, other oligomeric neurotoxic effects have been reported in the literature, such as endoplasmic reticulum stress, mitochondrial dysfunction and enhanced production of reactive oxygen species, eventually contributing to neural death (Alam et al., 2019; Ingelsson, 2016; Lashuel et al., 2013; Ono, 2017). This evidence suggests that, even if LBs and LNs represent the hallmarks of α-synucleinopathies, oligomers are strongly involved in the pathogenesis of these disorders. This supports synaptic dysfunction (and thus axonal synucleinopathy) as the primary insult of the neurodegenerative process, preceding cell death (Calo et al., 2016). Of note, several evidence supports the hypothesis that, starting from synapses, the neurodegenerative process spreads retrogradely to cell bodies (Calo et al., 2016). This is also in line with several studies on PD reporting that DA dysfunction precedes the development of Lewy pathology, as exemplified by the fact that the extent of striatum DA neurotransmission impairment and neurodegeneration considerably exceeds the neuronal degradation in the substantia nigra (SN) at the time of PD diagnosis (Calo et al., 2016; Caminiti et al., 2017).

Although several studies reported that the diffusion of pathological α-syn inclusions reflects the spreading of α-synuncleinopathies, the mechanisms underlying this process and its consequences continue to be disputed. The main hypothesis states that α-syn inclusions are transmitted from cell-to-cell by means of a prion-like mechanism (Calo et al., 2016; Goedert et al., 2017; Jan et al., 2021; Vargas et al., 2019). Prion (PRoteinaceous Infective ONly particle) are infectious agents composed of misfolded forms of proteins (called scarpie prion proteins, PrPSc) that may induce other normal prion proteins (PrPc) to misfold, thus causing a self-propagation of pathologic α-syn inclusions (Vargas et al., 2019). Since misfolded α-syn is structurally similar to PrPSc, several studies hypothesized that α-syn inclusions could follow the same spreading mechanism (Vargas et al., 2019). In this context, both in vitro and vivo studies demonstrated that α-syn fibrils

in the extracellular space have the potential to recruit unfolded α-syn monomers and, once uptake by connected cells, convert them into fibrils, thus seeding further misfolding and aggregation of native soluble α-syn aggregates (Jan et al., 2021; Vargas et al., 2019). Of note, it has also been reported that pathological α-syn inclusions are able to transfer not only through various cell types, but also through different mechanisms. In more details, misfolded α-syn is able to spread from neuron-to-neuron, from astrocyte-to-astrocyte, from neuron-to-astrocyte and from neuron-to-oligodendrocyte (Vargas et al., 2019). Moreover, based on the structural configuration adopted by α-syn, the spreading can take place through different mechanisms: i) monomers can transfer through exocytosis and passive diffusion, exosomes, autophagy; ii) oligomers can transfer through exocytosis, exosomes, endocytosis, tunneling nanotubes (TNTs); iii) fibrils can transfer through exosomes, endocytosis, lymphocyte-activation gene 3[1] (LAG3) endocytosis, TNTs.

Of note, the prion-like mechanism of α-syn spreading is consistent with the Braak staging hypothesis of PD, which categorizes the disease into six different stages based on α-syn pathology (Braak et al., 2003, 2004). Indeed, Braak and collaborators proposed that α-syn spreading involves the trans-synaptic transfer of pathological α-syn inclusions (see Figure 4) (Braak et al., 2003, 2004; Calo et al., 2016). Based on this hypothesis, this transfer follows a rostro-caudal direction characterized by the progressively involvement of supplementary brain regions with worsening of pathology in previously affected ones (Goedert et al., 2013). This spreading mechanism reflects the timing of onset, the type and the severity of both non-motor and motor symptoms which start years before the diagnosis and progressively evolve through the whole duration of the disease (Braak et al., 2003, 2004; Goedert et al., 2013; Vargas et al., 2019). In more detail, this process begins in the medulla oblongata (stage I), with first pathological α-syn inclusions appearing not only in the olfactory nucleus and olfactory bulb, but also in the dorsal motor nucleus of the glossopharyngeal (cranial nerve (CN) IX) and the vagus nerves (CN X), extending up to projections cells of the intermediate reticular zone (Braak et al., 2003). From here, α-syn pathology keeps spreading reaching the pontine tegmentum (stage II),

[1] Lymphocyte-activation gene 3 (LAG3) is a protein expressed on the surface of various immune cells (e.g., activated T cells, natural killer cells, B cells, and dendritic cells) where it regulates immune responses in order to prevent autoimmunity (Andrews et al., 2017). Of note, it has been demonstrated that LAG3 is able to selectively bind to α-syn fibrils, thus playing a crucial role in their spreading (Mao et al., 2016; Vargas et al., 2019).

with pathological α-syn inclusions appearing in the gigantocellular reticular nucleus and caudal raphe nuclei, also affecting projection neurons of the coeruleus-subcoeruleus complex (Braak et al., 2003). These initial stages reflect the prodromal phase of PD with main clinical manifestations being autonomic dysfunction (e.g., olfactory impairment, constipation, urinary and gastrointestinal symptoms), mood disorders and the emergence of RBD (Del Tredici & Braak, 2016; Poewe et al., 2017; Silva et al., 2023). However, with the exacerbation of α-syn pathology in previously affected areas and with the involvement of the midbrain, core motor manifestations of PD (i.e., bradykinesia, rigidity, rest tremor) also appear during stage III (Poewe et al., 2017; Silva et al., 2023). This is mainly due to the propagation of pathological α-syn inclusions to the SN pars compacta (SNpc) leading to the neurodegeneration of DA neurons (Braak et al., 2003). From here, pathological α-syn aggregates extend to the basal prosencephalon and mesocortex (stage IV), with lesions appearing in temporal mesocortex and allocortex (Braak et al., 2003). Finally, α-syn pathology spreads also to the neocortex leading to emergence of mild cognitive impairment (MCI) and, in the event of worst outcome, to dementia (Silva et al., 2023). Indeed, while during stage V pathological α-syn inclusions appear in high order association areas and prefrontal cortex (PFC); during stage VI, α-syn aggregates also manifest in first order sensory association areas and in the premotor cortex, eventually reaching primary sensory areas and primary motor fields (Braak et al., 2003).

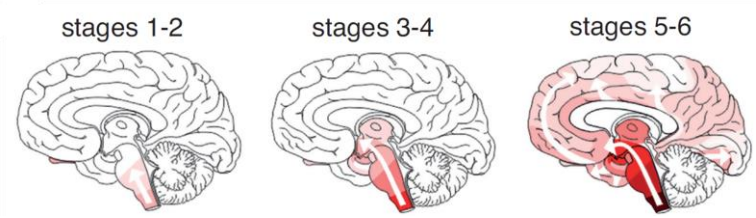


Figure 4. Schematic representation of the Braak staging hypothesis of PD showing the proposed brain regional distribution pattern of the lesions from stage I to stage VI. This figure was reprinted from Del Tredici & Braak, 2020 (Open Access Source). Of note, the severity of the pathology is indicated by darker degrees of shading.

Importantly, this staging hypothesis was updated in 2016 by Del Tredici and Braak (Del Tredici & Braak, 2016) suggesting that, based on experimental models, α-syn pathology starts in the enteric nervous system (ENS) and peripheric nervous system (PNS) by spreading via the CN X to the central nervous system (CNS). This gave rise to

the "*Dual-Hit Hypothesis*" which states that α-syn pathology begins in the ENS or PNS, specifically in the olfactory epithelium and/or in the gut mucosa in response to a combination of environmental and genetic factors (Jan et al., 2021; Troncoso-Escudero et al., 2018). Of note, this hypothesis is in line with the early appearance of non-motor symptoms in PD patients (Del Tredici & Braak, 2016; Goedert et al., 2013; Silva et al., 2023). Figures 5 illustrates the updated Braak staging hypothesis of PD.

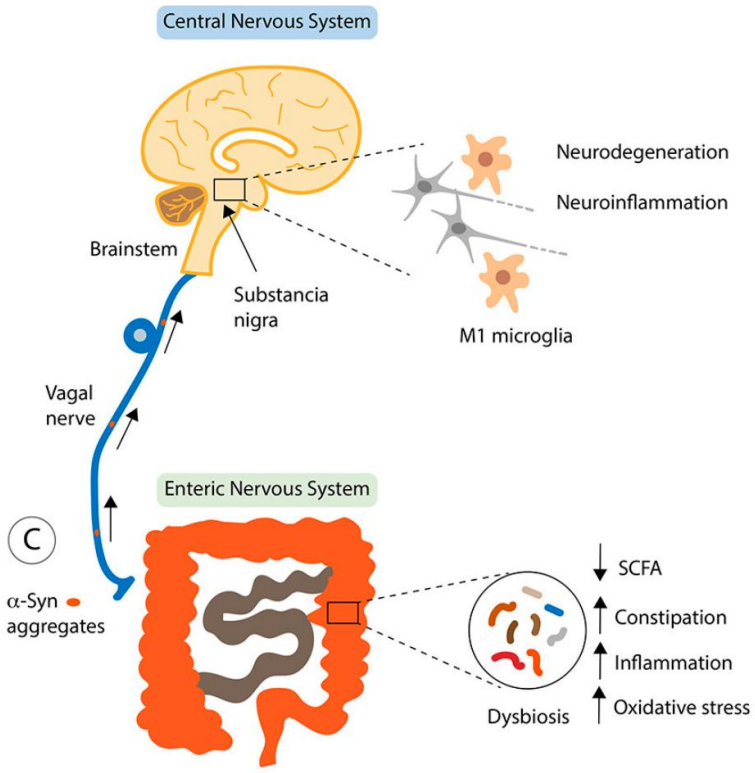


Figure 5. Schematic representation of the updated Braak staging hypothesis of PD. This figure was reprinted from Troncoso-Escudero et al., 2018 (Open Access Source). Specifically, the figure illustrates the gut-brain axis, related PD non-motor symptoms and environmental factors that might contribute to this process. Abbreviations: M1 microglia, pro-inflammatory reactive microglia phenotype; SCFA, Short chain fatty acids and vitamins; α-Syn, α-synuclein.

It is worth mentioning that the "*Dual-Hit Hypothesis*" is still matter of debate, thus leading to possible alternative hypothesis (Borghammer & Van Den Berge, 2019; Engelender & Isacson, 2017; Jan et al., 2021). In this context, a new perspective emerged when in 2018 Borghammer and collaborators suggested that LBs disorders (LBD) may arise following two different routes: the "*body-first*" (or gut-first) route and the "*brain-first*" route (see Figure 6) (Borghammer, 2018; Borghammer & Van Den Berge, 2019).

Those patients that follow the "*body-first*" route are characterized by a more severe autonomic dysfunction which manifests from the prodromal stages of the disorder, including a greater prevalence of RBD and hyposmia (Borghammer, 2021; Borghammer & Van Den Berge, 2019). This reflects the fact that this phenotype is believed to originate in the ENS and PNS and from there spreads to the CNS via the CN X, which is in line with the (updated) Braak staging hypothesis (Del Tredici & Braak, 2016). Of note, these patients are also prone to a faster non-motor, cognitive and motor progression of the disorder and to a symmetric motor profile which reflects a more symmetric loss of DAT (Borghammer, 2021; Borghammer & Van Den Berge, 2019). On the other hand, patients that follow the "*brain-first*" route are characterized by fewer autonomic symptoms reflecting a lesser damage to the ENS and PNS, thus leading to a marginal presence of RBD and hyposmia in prodromal phases (Borghammer, 2021; Borghammer & Van Den Berge, 2019). Moreover, these patients are usually characterized by a slower progression of the disorder with an asymmetric motor profile due to a more asymmetric neurodegeneration of DAT (Borghammer, 2021; Borghammer & Van Den Berge, 2019). This recent hypothesis may account for the heterogeneity of LBD symptoms which not only represents a hallmark of these disorders, but it also reflects differences in the underlying pathological process (Borghammer & Van Den Berge, 2019).

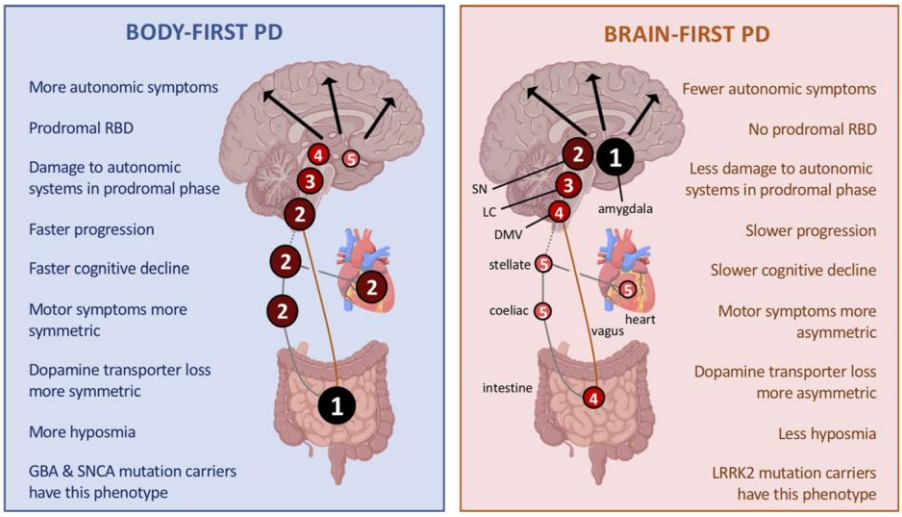


Figure 6. Schematic representation of the site of initial α-syn pathology (1) and the subsequent spatial-temporal sequence of progressive Lewy pathology (2-5). This figure was reprinted from Borghammer, 2021 (Open Access Source). The panel on the left illustrates the main characteristics of the "*body-first*" phenotype; meanwhile, the panel on the right shows the main features of the "*brain-first*" phenotype. Abbreviations: GBA, glucocerebrosidase; LRRK2,

leucine-rich repeat kinase 2; PD, Parkinson's Disease; RBD, rapid eye-movement sleep behavior disorder; SNCA, synuclein α.

1.1.2. Parkinson's Disease

Originally, PD was defined as "*shaking palsy*" (or Paralysis Agitans) due to the peculiar trait of involuntary tremulous motion, as first described by James Parkinson in 1817 (Parkinson, 2002). In his "An Essay on the shaking palsy" (Parkinson, 2002), James Parkinson described six patients apparently affected by PD, all characterized by five core symptoms: involuntary rest tremor, lessened muscular power, anteroflexion of the trunk, propulsion, undisturbed senses and intellect (Hesselink, 1996). From there, concepts and definitions of PD evolved reaching a turning point in 1872, when Jean-Martin Charcot (Charcot, 1872) differentiated the three cardinal motor symptoms of PD: rest tremor, rigidity and bradykinesia (Goetz, 2011; Hesselink, 1996). Moreover, Jean-Martin Charcot was also able to distinguish PD from other tremor-related disorders (i.e., multiple sclerosis) and to identify different PD phenotypes (i.e., tremor-dominant versus akinetic/rigid dominant) (Charcot, 1872; Goetz, 2011).

Since its first description, the course of PD has been delineated by a slow progression marked by both non-motor and motor manifestations, which may arise long before the core triad symptoms as exemplified by the difficulties of patients in performing simple everyday activities (Charcot, 1872; Goetz, 2011; Hesselink, 1996; Parkinson, 2002). These observations strongly reflect the heterogeneity of PD symptoms and progression, which is crucial to account for when studying disease clinical trajectories, as described in the next paragraphs.

1.1.2.1. Epidemiology of PD

The World Health Organization (WHO) stated that PD prevalence has doubled in the last 25 years, globally affecting over 8,5 million individuals in 2019 (WHO, 2023). In 2021, the Global Burden for Disease (GBD) resource center conducted a systematic analysis on the global burden of PD from 1990 to 2016 (Ou et al., 2021) reporting that nowadays neurological disorders are the leading cause of handicap, and that PD represents the fastest growing of these disorders. These data have prompted the introduction of the term "*Parkinson pandemic*" which highlights the emergency of the

situation; indeed, current estimates suggest that at least 12,9 million individuals will have PD by 2040 (Dorsey & Bloem, 2018). In this context, the study published by Ou and collaborators in 2021 (Ou et al., 2021) offers a comprehensive overview of the growing global burden of PD, outlining the latest global trends concerning its incidence and prevalence. The authors reported an increase in both global incidence and prevalence of PD between 1990 and 2019. By 2019, the global age-standardized rate (ASR) of the incidence was 13.43/100,000 person/years (159.73% increase since 1990); meanwhile, the global ASR of the prevalence was 106.28/100,000 person/year (155.50% increase from 1990) (Ou et al., 2021). Of note, both incidence and prevalence were higher for males which showed an ASR of the incidence of 17.79/100,000 person/year (vs females: 9.95/100,000 person/year) and an ASR of the prevalence of 129.33/100,000 person/year (vs females: 87.59/100,000 person/year) (Ou et al., 2021). Concerning differences in age groups, highest incident and prevalence numbers were observed in individuals aged over 65 years (Ou et al., 2021). Indeed, it is worth noting that PD is an age-related disease usually diagnosed after age 60 (NIA, 2022). These data align with epidemiological studies on PD reporting that both advanced age and male sex are risk factors for PD (Ben-Shlomo et al., 2024; Bloem et al., 2021). In this regard, different factors have been hypothesized to contribute to PD male predominance, such as a greater exposure of men to adverse environmental risk factors (i.e., pesticides) and the possible protective effect of sex hormones against neurodegeneration thanks to their antioxidant properties (Ben-Shlomo et al., 2024; De Lau & Breteler, 2006). Apart from these, in the literature, other environmental, socio-behavioral and genetic risk factors have been reported to contribute to PD pathogenesis. In more detail, exposure to pesticides (e.g., paraquat, rotenone, maneb, benomyl, organochlorines) and chlorinated solvents, jobs linked to toxicant exposure (e.g., farming, welding), high dietary consumption of dairy products, reduced estrogen status (in women), history of melanoma and traumatic brain injury are the main environmental and socio-behavioral factors heightening the risk of PD (Ascherio & Schwarzschild, 2016; Ben-Shlomo et al., 2024). On the contrary, factors such as cigarette smoking, caffeine consumption, greater physical activity, higher serum urate concentrations, use of ibuprofen and other common medications were linked to a diminished risk of developing PD (Ascherio & Schwarzschild, 2016; Ben-Shlomo et al., 2024).

Concerning genetics, the heritability of PD has been estimated to range between 22% and 40% (Morris et al., 2024). Of note, while some individuals exhibit genetic mutations related to familial forms of the disease, several patients present multiple common genetic variants (i.e., a polygenic risk profile) which heighten the risk of developing PD (Morris et al., 2024). The main genetic mutations identified as having a causal association with PD are the autosomal dominant genes synuclein α (SNCA), leucine-rich repeat kinase 2 (LRRK2), vacuolar protein sorting ortholog 35 (VPS35), and the autosomal recessive genes parkin (PRKN), phosphatase and tensin homolog-induced kinase 1 (PINK1), PD protein 7 (DJ-1), dnaJ heat shock protein family 40 member C6 (DNAJC6), vacuolar protein sorting 13 homolog C (VPS13C) (Fernandez-Santiago & Sharma, 2022; Morris et al., 2024). Specifically, PRKN, PINK1 and DJ-1 have been linked to early-onset and juvenile PD forms (Fernandez-Santiago & Sharma, 2022). Lastly, genetic variants in genes encoding the enzyme glucocerebrosidase (GBA) and the microtubule-associated protein Tau (MAPT) are identified as some of the most significant risk determinants for PD (Fernandez-Santiago & Sharma, 2022).

1.1.2.2. Diagnosis, Clinical Features and Subtyping of PD

In 2015 the international PD and Movement Disorder Society (MDS) published a review article aiming to outline the official clinical criteria for PD diagnosis (Postuma et al., 2015). Importantly, these criteria can only be applied in the presence of parkinsonism, which is referred to as bradykinesia, along with rest tremor and/or rigidity (Postuma et al., 2015). These three symptoms are PD cardinal motor manifestations, and they have been extensively described within the MDS statement. Bradykinesia is defined as a reduction in movement amplitude and speed, leading to a noticeable slowness in motion, which becomes more pronounced as the activity progresses (Postuma et al., 2015). The term “lead pipe resistance” is usually used to refer to PD rigidity by describing a constant resistance to passive movement (not merely indicative of an inability to relax) (Postuma et al., 2015). Finally, MDS criteria defined rest tremor as a 4-6 Hz tremor in a completely at-rest limb, which diminishes/disappears when the limb initiates movement (Postuma et al., 2015). The possible presence of these symptoms is usually carried out using the MDS Unified PD Rating Scale (UPDRS) (Goetz et al., 2008), that is a standardized tool created by Goetz and colleagues for assessing PD severity and progression. Once parkinsonism

has been determined, in order to establish a clinical diagnosis of PD, it is mandatory: i) to prove the absence of absolute exclusion criteria; ii) to check for the presence of at least two supportive criteria; and iii) to verify the absence of red flags (see Table 1 for a complete overview) (Postuma et al., 2015).

A) Absolute Exclusion Criteria	1. Presence of unequivocal cerebellar abnormalities.
	2. Presence of downward vertical supranuclear gaze palsy, or selective slowing of downward vertical saccades.
	3. Diagnosis of probable behavioral variant frontotemporal dementia/primary progressive aphasia within the first 5 years of disease.
	4. Presence of parkinsonian features restricted to the lower limbs >3 years.
	5. Drug-induced parkinsonism (due to DA receptor blocker/DA-depleting agents).
	6. Even in the presence of a moderate severity of the disease, absence of observable response to high-dose levodopa.
	7. Presence of unequivocal cortical sensory loss, clear limb ideomotor apraxia, or progressive aphasia.
	8. Normal functional neuroimaging of the presynaptic dopaminergic system.
	9. Presence of an alternative condition which parkinsonism is more likely than PD or which is known to produce parkinsonism.
B) Supportive Criteria	1. Presence of significant beneficial response to DA therapy.
	2. Presence of levodopa-induced dyskinesia.
	3. Presence of rest tremor of a limb.
	4. Positive ancillary diagnostic tests (specificity > 80% for differential diagnosis of PD): i) olfactory tests documenting hyposmia; ii) metaiodobenzylguanidine scintigraphy documenting cardiac sympathetic denervation.
C) Red Flags	1. Rapid progression of gait impairment requiring regular use of wheelchair within 5 years of onset.
	2. A complete absence of progression of motor symptoms over ≥ 5 years (unless stability is related to treatment).
	3. Presence of early bulbar dysfunction within the first 5 years of disease.
	4. Presence of inspiratory respiratory dysfunction
	5. Presence of severe autonomic failure in the first 5 years of disease.
	6. Presence of recurrent falls (i.e., for more than 1 year) because of impaired balance within 3 years of onset.
	7. Presence of disproportionate anterocollis or contractures of hand/feet within the first 10 years of disease.
	8. Absence of nonmotor symptoms despite 5 years of disease duration (i.e., sleep dysfunction, autonomic dysfunction, hyposmia, psychiatric dysfunction)
	9. Presence of otherwise unexplained pyramidal tract signs.
	10. Presence of bilateral symmetric parkinsonism throughout the disease course (i.e., bilateral symptom onset with no side predominance).

Table 1. Summary of the MDS absolute exclusion criteria, supportive criteria and red flags for diagnosis of PD. These tables were created by me based on Postuma et al., 2015. A) Panel A summarizes MDS absolute exclusion criteria for PD diagnosis (Postuma et al., 2015). B) Panel B summarizes MDS supportive criteria for PD diagnosis (Postuma et al., 2015). C) Panel C

summarizes MDS red flags for PD diagnosis (Postuma et al., 2015). Abbreviations: DA, dopamine; PD, Parkinson's Disease.

Also freezing of gait (FoG) phenomenon and camptocormia fall within the category of PD motor symptoms. Concerning FoG, it is described as intermittent stops or a significant decrease in the forward motion of the feet, that goes beyond the person's intention to walk (Nutt et al., 2011; Weiss et al., 2020). Moreover, PD patients usually present the typical camptocormic posture characterized by a pronounced forward bending of the trunk which worsens while walking and shows little to no improvement with L-DOPA treatment (Abe et al., 2010). This complex spectrum of motor symptoms is further aggravated by the presence of different non-motor features which can appear years before clinical motor manifestations, thus enabling the identification of those patients with a high risk of developing PD. Several studies reported that hyposmia, constipation, iRBD, anxiety and depression are among the main risk factors for PD in healthy individuals (Antonini et al., 2012; Bugalho et al., 2021; Poewe, 2008; Poewe et al., 2017; Poewe & Mahlknecht, 2009). Indeed, these symptoms usually emerge throughout PD prodromal phase and reflect the ongoing underlying neuropathological process related to α-syn spreading (Braak stages I and II). Nevertheless, disability caused by non-motor symptoms only emerges in later phases of the disease, once α-syn pathology in previously affected regions worsens (Poewe & Mahlknecht, 2009). In 2008, Hely and colleagues published the last update of a longitudinal study conducted on 136 patients with PD who were followed over time for 20 years (Hely et al., 2008). Of note, the percentage of patients showing non-motor manifestations gradually rose throughout the follow-up. After 20 years, despite the fact that 100 out of 136 patients have died, 48% of patients experienced orthostatic hypotension (OH), 71% urinary incontinence, 74% hallucinations and 80% dementia (Hely et al., 2008). These data support the evidence that PD progression is very heterogeneous, with non-motor symptoms playing a crucial role in PD clinical trajectories and with dementia representing the worst possible outcome (Poewe et al., 2017). Based on these facts, several studies suggested the subtyping of PD based on different clinical features to gain new insights into the underlying neuropathological mechanisms and the prognosis of the disease (Fereshtehnejad & Postuma, 2017; Mestre et al., 2021; Thenganatt & Jankovic, 2014; Van Rooden et al., 2010; van Rooden et al., 2011).

Different methodological approaches have been employed for the classification of PD subtypes: i) a priori hypothesis approach (e.g., based on age of onset, motor features, cognitive profile, non-motor characteristics); ii) data-driven approach (i.e., cluster analysis (CA)); and iii) biomarker-based approach (e.g., blood, cerebrospinal fluid (CSF), genetic, and imaging biomarkers) (Fereshtehnejad & Postuma, 2017; Thenganatt & Jankovic, 2014). Among a priori hypothesis approaches, one of the most widely studied classification methods in the literature is the motor-based one, which enabled the identification of two main PD phenotypes: 1) tremor-dominant PD; and 2) postural instability and gait difficulties (PIGD) PD (Thenganatt & Jankovic, 2014). On one hand, the tremor-dominant PD phenotype has been associated with a good prognosis and a slow progression (mainly related to tremor) (Thenganatt & Jankovic, 2014). These patients show predominant rest tremor, mild non-tremor signs, good response to L-DOPA; additionally, these patients usually present the typical "eagle wing" configuration on iodine 123-radiolabeled 2β-carbomethoxy-3β-(4-iodophenyl)-N-(3-fluoropropyl) nortropane (^{123}I-FP-CIT) single photon emission-computed tomography (SPECT) – also known as Dopamine Transporter SPECT (DAT-SPECT) (see Figure 7A) (Murakami et al., 2018; Thenganatt & Jankovic, 2014). On the other hand, PIGD PD phenotype has been attributed to poor prognosis and fast progression (Thenganatt & Jankovic, 2014). These patients show predominant bradykinesia and rigidity and are marked by a high prevalence of non-motor features (i.e., dementia, depression, anosmia) which heighten the severity of the disorder (Thenganatt & Jankovic, 2014). Moreover, these patients usually present the typical "egg-shaped" configuration on DAT-SPECT (see Figure 7B) (Murakami et al., 2018; Thenganatt & Jankovic, 2014).

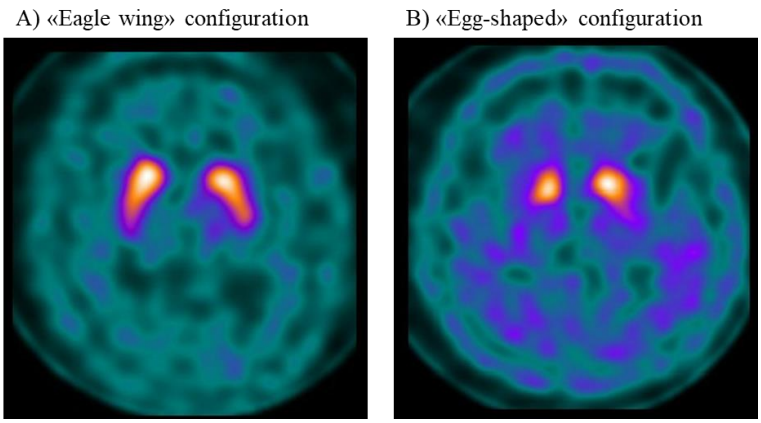


Figure 7. Examples of "eagle wing" (A) and "egg-shaped" (B) configurations on DAT-SPECT in drug-naïve PD patients. This figure was reprinted from Murakami et al., 2018 (Open Access Source).

However, subtyping PD patients based on a single feature (e.g., motor symptoms) has several limitations. To overcome these, various studies have employed data-driven approaches, which have the significant advantage of not relying on a priori hypotheses. Among these, CA is the most commonly used methodology. CA is an unsupervised learning statistical technique used to categorize individuals into groups (i.e., clusters) with minimal intra-group differences, and substantial inter-group differences (Fereshtehnejad & Postuma, 2017; Van Rooden et al., 2010). In 2011 Van Rooden and colleagues (van Rooden et al., 2011) published a study applying CA to a broad spectrum of motor and non-motor manifestations in 802 PD patients. From the results, four clusters emerged characterized by differences in the severity of nondopaminergic features and motor complications: cluster 1) mildly affect in both domains; cluster 2) characterized by severe motor complications; cluster 3) characterized by severe nondopaminergic features; cluster 4) severely affected in both domains (van Rooden et al., 2011). Another study published in 2017 by Fereshtehnejad and colleagues (Fereshtehnejad et al., 2017) applied CA to a comprehensive baseline dataset (including clinical, neuroimaging, biospecimen and genetic info) of 421 PD patients. From these data, the authors derived three clusters: cluster 1) "mild motor-predominant" with less severe scores in both motor and non-motor domain; cluster 2) "intermediate" with intermediate scores in both motor and non-motor domains; cluster 3) "diffuse malignant" with worst scores in both motor and non-motor domains (Fereshtehnejad et al., 2017). Notably, cluster 4 from the study by van Rooden and colleagues (van Rooden et al., 2011) and cluster 3 from the study by Fereshtehnejad and colleagues (Fereshtehnejad et al., 2017) reflected a more malignant PD phenotype, characterized by an earlier and more severe onset of motor and non-motor manifestations, greater occurrence of cognitive deficits, and a fast disease advancement. This more severe PD phenotype also aligns with the previously mentioned PIGD PD phenotype identified using a priori hypothesis motor-based approach. Altogether, these data suggest that those patients exhibiting this malignant PD phenotype face a worse prognosis, with a heightened risk of dementia as the disease progresses. Nevertheless, this approach presents several limitations such as lack of comprehensive data in many studies, sample

selection bias, lack of longitudinal data (thus preventing validation of disease progression), the inclusion of patients with different disease durations, lastly individuals within the same cluster may not exhibit all defining features (Fereshtehnejad & Postuma, 2017). Moreover, the use of different variables across studies has resulted in clusters that are highly heterogeneous and frequently controversial (Fereshtehnejad & Postuma, 2017). For these reasons, few studies proposed a biomarker-based approach for subtyping of PD (Fereshtehnejad & Postuma, 2017; Kang et al., 2016; Lücking et al., 2000; Thenganatt & Jankovic, 2014; Wu et al., 2011). This new approach has the main advantage of identifying subtypes at early stages of the disease, thus enabling the development of potential tailored therapeutic strategies based on specific biomarker profiles before the neurodegenerative process is too advanced (Fereshtehnejad & Postuma, 2017). Indeed, the identification (and combination) of risk factors and markers able to determine which patients are likely to transition to a more malignant PD phenotype characterized by the development of dementia is a research priority. The next paragraph will outline the main risk factors for malignant PD.

1.1.2.3. Risk factors for malignant PD

As described in detail above, PD patients may follow different clinical trajectories from a milder to a more severe form of the disease with dementia representing the worst possible outcome. These data strongly support the value of studying risk factors for the progression to dementia; indeed, timely diagnosis of PDD could open doors for early intervention and innovative treatments, potentially improving patient outcomes and survival rates (Xu et al., 2016). In the literature, several risk factors have been identified, such as advanced age, male sex, low education, advanced disease stage, PIGD phenotype, genetic variants, hallucinations, iRBD, dysautonomia and MCI (Vasconcellos & Pereira, 2015; Xu et al., 2016). These latter three seem to guide patients' path towards PDD.

Notably, iRBD is not only a risk factor for the conversion to a malignant form of PD and the future progression to PDD (Postuma et al., 2012), but it is considered a prodromal stage of α-synucleinopathies (Galbiati et al., 2019). This complex relationship will be deeply described in paragraph 1.2.4.

Concerning autonomic dysfunction, it has been previously showed that PD is marked by a broad spectrum of non-motor manifestations, some of them characterized by primary

autonomic failure such as OH, other cardiovascular irregularities, anhidrosis, heat intolerance, constipation, dysphagia, urinary bladder, nocturia, incontinence, erectile and ejaculatory failure, anisocoria, Horner's syndrome, stridor, inspiratory gasps, apneic episodes (Poewe, 2007). Of note, OH and cardiac sympathetic denervation have been specifically linked to cognitive impairment and PDD. In this context, several studies hypothesized a relationship between OH and cognitive deficits in PD; however, results were highly heterogenous (Allcock et al., 2006; Loureiro et al., 2023; McDonald et al., 2016; Ruiz Barrio et al., 2023). In order to explain this nexus, different mechanisms have been discussed (McDonald et al., 2016; Ruiz Barrio et al., 2023). First, OH causes chronic hypoxia due to repeated episodes of cerebral hypoperfusion, which potentially results in subcortical ischemic damage. Concurrently, supine hypertension may lead to vascular damage. The buildup of these damages may ultimately contribute to cognitive decline (McDonald et al., 2016; Ruiz Barrio et al., 2023). Second, based on Braak staging hypothesis, spreading of α-syn pathology from the ENS and PNS to the CNS may explain why OH often emerge before cognitive deficits (McDonald et al., 2016). Third, the association between OH and cognitive impairment may emerge from central and peripheral noradrenergic dysfunction (McDonald et al., 2016). Of note, a recent study published by Ruiz Barrio and colleagues (Ruiz Barrio et al., 2023) on 132 PD patients found a significant association between OH, cognitive impairment and future development of dementia which was not explained by the severity of α-syn, Aβ, tau, or cerebrovascular pathologies. The authors suggested that these findings may support chronic hypoxia-related neurodegeneration as a major factor contributing to cognitive decline (Ruiz Barrio et al., 2023). Besides OH, cardiac sympathetic denervation has been identified as the other main autonomic-related dysfunction related to cognitive deficits and dementia in PD. This has been studied in the literature using 123iodine-metaiodobenzylguanidine SPECT (^{123}I-MIBG-SPECT), a neuroimaging technique that allows the evaluation of postganglionic presynaptic cardiac sympathetic nerve endings, thus representing an index of cardiovascular functioning (Cascini et al., 2013; Yamashina & Yamazaki, 2007). In this context, prior research suggested the usefulness of ^{123}I-MIBG-SPECT in the differential diagnosis between neurodegenerative diseases (Oide et al., 2003; Treglia et al., 2012; Treglia & Cason, 2012; Yoshita et al., 2006). Moreover, ^{123}I-MIBG-SPECT is also considered an early biomarker of dysautonomia in PD (Orimo

et al., 2007, 2012). Specifically, Kim and colleagues demonstrated that a more severe cardiac sympathetic denervation in PD patients was associated with a worse cognitive profile and an increased risk of dementia (Kim et al., 2009).

Finally, cognitive impairment in PD is common, often begin early, and vary widely; moreover, MCI has been recognized as a risk determinant for PDD (Baiano et al., 2020; Hoogland et al., 2017). A recent meta-analysis reported that 25% of PD patients developed MCI (PD+MCI) within three years from the diagnosis (Saredakis et al., 2019); however, this rate varied greatly across studies due to both demographic and clinical confounding factors (Baiano et al., 2020). Moreover, both neuropsychiatric symptoms (i.e., depression, apathy, and anxiety) and PIGD phenotype have been reported to have a higher association with PD+MCI with respect to PD patients without MCI (PD-MCI) (Baiano et al., 2020). Thus, in order to identify those patients with a heightened risk of dementia, different studies tried to characterized PD cognitive profile (Aarsland et al., 2021; Curtis et al., 2019). Indeed, cognitive changes in PD patients have been observed in several domains, such as executive function (Kudlicka et al., 2011), attention (Dujardin et al., 2013), verbal memory (Assogna et al., 2010; Bohlhalter et al., 2009; Galtier et al., 2014; Hanoğlu et al., 2019), language (Liu et al., 2015) and visuospatial abilities (Chastan et al., 2019; Gullett et al., 2013; Montse et al., 2001). Given these premises, with the aim of disentangling the heterogeneity characterizing cognitive changes in PD+MCI patients, Kehagia and colleagues proposed the "*Dual Syndrome Hypothesis*" (Kehagia et al., 2012). This hypothesis states that, based on the progression of PD cognitive changes, two different phenotypes can be identified: phenotype 1) patients exhibiting a tremor-dominant profile with cognitive deficits mainly affecting the executive function domain due to a frontostriatal dopaminergic dysfunction. These patients are characterized by a slow progression of cognitive impairment (Kehagia et al., 2012); phenotype 2) patients exhibiting a PIGD profile with psychiatric manifestations and severe cognitive deficits mainly affecting visuospatial and memory domains due to a posterior-cortical impairment. These patients rapidly progress to dementia as a result of a supplementary cholinergic dysfunction (Kehagia et al., 2012). This hypothesis is supported by several findings showing that, although cognitive changes in PD patients are influenced by both DA and cholinergic deficiency, this latter is a crucial factor for the development of dementia (Klein et al., 2010; Müller & Bohnen, 2013; Shimada et al., 2009). Indeed, PDD

seems to be mainly linked to a posterior cholinergic dysfunction resulting in a more severe involvement of visuospatial abilities and memory cognitive domains (Kehagia et al., 2012).

1.1.2.4. PDD: clinical criteria and differential diagnosis

The GBD 2019 study (GBD 2021 Causes of Death Collaborators, 2021) estimated that PD accounted for 1,71 million of dementia cases globally (second only to dementia cases due to stroke). Indeed, a recent meta-analysis published by Severiano e Sousa and colleagues (Severiano e Sousa et al., 2022) estimated the global frequency of PDD reporting that the 26.3% of PD patients were diagnosed with dementia. Notably, this prevalence increased with disease duration (and age); indeed, frequency of dementia in patients aged 60-75 in early stages of PD (5-10 years of disease duration) was 16-20%, in patients of the same age in advanced stages of PD (10-15 years of disease duration) was 20-30%, and in patients aged over 75 in late stages of PD (> 15 years of disease duration) was 50-60% (Severiano e Sousa et al., 2022). Given these premises, clinical standards for PDD diagnosis have been recommended (Emre et al., 2007). These involves diagnosis of PD, followed by a diagnosis of dementia marked by an insidious onset and a gradual advancement (Emre et al., 2007). Based on history, clinical and mental examinations, dementia must be proven to entail: 1) decline in more than one cognitive domain; 2) deterioration relative to a former level of functioning; 3) this cognitive decline must be sufficiently severe to affect patients' daily life (independent of motor and autonomic complications) (Emre et al., 2007). In more detail, the MDS task force which has been charged to define the criteria, also tried to identify the main clinical features associated with PDD. Specifically, the authors suggested that PDD is characterized by deficits in multiple cognitive domains (i.e., attention, executive function, visuospatial abilities, memory, language – as deeply described in paragraph 1.1.2.) and by different behavioral manifestations (i.e., apathy, alterations in personality and mood, hallucinations, delusions, excessive daytime sleepiness) (Emre et al., 2007).

The rapid cognitive decline seen in PDD shares clinical similarities with DLB, raising questions about whether these conditions are distinct or represent overlapping pathological entities (Friedman, 2018; Jellinger, 2018, 2022; Jellinger & Korczyn, 2018; Tsuboi & Dickson, 2005). Indeed, the fourth consensus report of the DLB Consortium

(McKeith et al., 2017) stated that, beyond the presence of dementia[2], the core clinical criteria for DLB diagnosis are: 1) cognitive fluctuations; 2) visual hallucinations; 3) RBD; 4) one or more core motor manifestations of parkinsonism (i.e., bradykinesia, rest tremor, or rigidity). Notably, among supportive clinical features for DLB diagnosis are listed several motor (i.e., postural instability, repeated falls), non-motor (syncope or other transient episodes of unresponsiveness, hypersomnia, hyposmia, severe autonomic dysfunction – constipation, OH, urinary incontinence) and psychiatric (i.e., other types of hallucinations, delusions, mood alterations) manifestations (McKeith et al., 2017) which represent hallmarks of PD and, more specifically, of PDD. Moreover, the fourth consensus report of the DLB Consortium (McKeith et al., 2017) also identified several indicative and supportive biomarkers for DLB diagnosis. As concerns indicative biomarkers, reduced DAT uptake in basal ganglia on DAT-SPECT or positron emission tomography (PET), reduced MIBG uptake on ^{123}I-MIBG-SPECT, REM sleep without atonia (RSWA) on polysomnography (PSG) have been determined (McKeith et al., 2017). With regard to supportive biomarkers, relative preservation of medial temporal lobe structures on computed tomography (CT) or magnetic resonance imaging (MRI), reduced occipital activity on SPECT/PET, cingulate island sign[3] on 18fluorodeoxyglucose PET ([^{18}F]FDG-PET), posterior slow-waves (SW) activity on electroencephalography (EEG) have been pinpointed (McKeith et al., 2017). Nowadays, the only defined criteria to differentiate PDD and DLB is the so-called "one-year rule", which states that in order to diagnose DLB one year must pass between the onset of dementia and parkinsonism (vice-versa for PDD) (McKeith et al., 2017). However, since cognitive decline has been documented to begin years or even decades prior to PD diagnosis, the "one-year rule" may not be the most reliable method for differentiating between the two disorders (Jellinger, 2018; Jellinger & Korczyn, 2018). Indeed, PDD and DLB share striking similarities in their clinical, neuropathological, genetic,

[2] Dementia [definition based on the fourth consensus report of the DLB Consortium (McKeith et al., 2017)]: progressive cognitive decline severe enough to affect social, occupational and instrumental activities of daily living (IADL). Deficits involve several cognitive domains such as memory, attention, executive function and visuospatial abilities.

[3] Cingulate island sign (CIS): is a radiological finding observed using [^{18}F]FDG-PET in patients with DLB and it refers to a relative sparing of the posterior cingulate cortex as compared to the precuneus plus cuneus (Iizuka et al., 2017). CIS is particularly useful in the differential diagnosis with Alzheimer's disease (AD), where it is typically not observed.

neuropsychological and neuropsychiatric profiles (see Table 2) (Jellinger, 2018; Jellinger & Korczyn, 2018). Regarding neuropsychological and neuropsychiatric features, these include impairments in executive function, planning, abstract thinking, multitasking and visuospatial abilities, along with symptoms like cognitive fluctuations, depression, anxiety, apathy, delusions and hallucinations (Friedman, 2018). As dementia progresses, also deficits in memory, language and praxis domains appear (Friedman, 2018). Both conditions resembling neuropathological traits, such as widespread LBs, making them indistinguishable on a pathological level (Friedman, 2018; Jellinger, 2022; Lippa et al., 2007). For all these reasons, it has been hypothesized in the literature that PDD and DLB may represent subtypes along the same LBD spectrum, with DLB at the more severe end near AD (Jellinger, 2018; Jellinger & Korczyn, 2018) and iRBD at the beginning of this continuum.

Category	Similarities between PDD and DLB
Clinical Features	• Motor symptoms: rigidity, akinesia • Neuropsychological profile: deficits in executive functions, planning, abstract thinking, multitasking and visuospatial abilities (later in memory, language, praxis) • Neuropsychiatric profile: mood disorders (i.e., depression, anxiety, apathy), cognitive fluctuations, delusions and hallucinations • Non-motor symptoms: autonomic dysfunction, RBD
Neuroimaging and Electrophysiological Features	• Reduced DAT uptake in basal ganglia on DAT-SPECT • Reduced MIBG uptake on ^{123}I-MIBG-SPECT • Relative preservation of the medial temporal lobe structures on CT/MRI • Occipital hypometabolism on SPECT/PET • Posterior SW activity on EEG
Genetic Features	• GBA mutations • MAPT mutations • APOE mutations
Morphological Features	• Cortical and subcortical α-syn pathology and AD-related pathology • Similar Braak LBs stages (4-6) and LNs stages (5 or 6) • Relationship between α-syn and tau aggregation to Aβ deposition in frontal and temporal cortex • Synaptic dysfunction due to α-syn pathology in the presynapses

Table 2. Summary of the main similarities between PDD and DLB. This table was created by me based on Jellinger et al., 2018. Abbreviations: ^{123}I-MIBG-SPECT, 123iodine-metaiodobenzylguanidine single photon emission-computed tomography; Aβ, Amyloid-β; AD, Alzheimer's Disease; APOE, Apolipoprotein E; CT, Computed tomography; DAT, Dopamine Transporter; DAT-SPECT, Dopamine Transporter single photon emission-computed tomography; DLB, Dementia with Lewy Bodies; EEG, Electroencephalography; GBA, Glucocerebrosidase; LBs, Lewy bodies; LNs, Lewy neurites; MAPT, Microtubule-associated protein Tau; MIBG, Metaiodobenzylguanidine; MRI, Magnetic resonance imaging; PDD, Parkinson's Disease with Dementia; PET, Positron emission tomography; RBD, Rapid Eye-Movement Sleep Behavior Disorder; SPECT, Single photon emission-computed tomography; SW, Slow wave; α-syn, α-synuclein.

1.2. Isolated Rapid Eye-Movement Sleep Behavior Disorder

The movie "Cinderella" created by Walt Disney in 1950 is famous worldwide. In a well-known scene of that movie, the dog Bruno is dreaming about chasing his enemy, the cat Lucifer. Bruno is asleep but also enacting his nightmare by barking and mimicking running. This is considered the first cinematic representation of RBD (Janković et al., 2006). However, the first scientific description of the disorder can be traced to 1986 when Carlos H. Schenck and colleagues (Schenck et al., 1986) observed that certain patients presented a history of aggressive behaviors during sleep harming themselves and/or their bed partners. PSG recording documented that these behaviors used to occur during REM sleep, which was marked by loss of muscle atonia and increased REM ocular activity and density (Schenck et al., 1986). These behaviors were found to be associated with vivid and often frightening dreams, as if those patients were acting out what they were dreaming (Schenck et al., 1986). Since its first movie adaptation and scientific description, the concept of RBD evolved. It has been distinguished between primary or idiopathic/isolated iRBD, when it presents in the absence of any other disorder, and secondary/symptomatic RBD (sRBD), when it presents following other neurological diseases (i.e., PD, DLB, or multiple system atrophy (MSA)) (St Louis & Boeve, 2017). Nowadays, iRBD is considered a prodromal stage of neurodegenerative disorders, primarily α-synucleinopathies (Galbiati et al., 2019). In light of this evidence, over time, the term "idiopathic" has been replaced with the term "isolated" RBD, reflecting both its status as a clinically isolated condition and its role as an early manifestation of a neurodegenerative disorder (Högl et al., 2018). In the next paragraphs, a comprehensive overview of iRBD will be provided.

1.2.1. Epidemiology of iRBD

A definitive diagnosis of iRBD can only be achieved through PSG, which is an expensive, time-consuming and not always accessible tool (Dauvilliers et al., 2018). Over time, several questionnaires for the screening of iRBD have been developed; however, these only allow for a probable diagnosis of the disorder (Dauvilliers et al., 2018). Even though the exact prevalence and incidence of the disorder in the general population remain unknown, both PSG- and questionnaire-based studies tried to provide the most accurate estimates possible. In this context, a recent meta-analysis published by Cicero

and colleagues in 2021 (Cicero et al., 2021) aimed to compute the overall estimates of the prevalence of definitive and probable iRBD. On one hand, questionnaire-based studies reported a prevalence of probable iRBD ranging between 0.57-13.58% resulting in a pooled prevalence of 5.65% (Aye et al., 2020; Baldin et al., 2020; Boot et al., 2012; Cicero et al., 2021; Hattori et al., 2020; Ma et al., 2016, 2017; Mahlknecht et al., 2015; Nomura et al., 2015; Noyce et al., 2014; Shprecher et al., 2019; Shprecher et al., 2020; Uemura et al., 2011; Wong et al., 2016; Yao et al., 2019). On the other hand, PSG-based studies reported a prevalence of definite iRBD which ranges between 0.29-1.15% resulting in a pooled prevalence of 0.68% (Chiu et al., 2000; Cicero et al., 2021; Haba-Rubio et al., 2018; Kang et al., 2013; Pujol et al., 2017; Sasai-Sakuma et al., 2020). These findings highlight how questionnaire-based studies highly overestimate iRBD prevalence by including several false-positive cases which are probably related to other conditions with a similar clinical presentation, such as non-REM (NREM) parasomnias, obstructive sleep apnea (OSA) or periodic limb movement (PLM) disorders (Dauvilliers et al., 2018).

Concerning the age of onset of the disorder, diagnosis of iRBD most frequently occurs > 50 years (range: 52-65 years) with a strong male predominance (between 70-87.5 %) and with symptoms presenting between 45-61 years (Kryger et al., 2017; Olson et al., 2000; Schenck et al., 1993; Takeuchi et al., 2020; Wing et al., 2008; Zhou et al., 2015). Indeed, as for PD, epidemiological studies reported that both advanced age and male sex represent risk factors for iRBD (Dauvilliers et al., 2018; Wu et al., 2022). The main reason behind iRBD male predominance is ascribed to the clinical presentation of the disorder. Indeed, it has been hypothesized that iRBD may be under-recognized in females because women with iRBD tend to show less aggressive dream-enacting behaviors compared to men, resulting in less sleep-related injuries. For this reason, female patients might seek clinical help less frequently (Dauvilliers et al., 2018; Fernández-Arcos et al., 2016; Kryger et al., 2017; Schenck et al., 2019). It is worth noting that a recent study published in 2020 by Takeuchi and collaborators tried to characterize female and male iRBD. The authors observed that, even though female patients with iRBD had a later symptoms onset and a higher phasic electromyographic (EMG) activity, the progression of the disease was similar to that in male patients (Takeuchi et al., 2020).

Regarding iRBD genetics, it only shares some common features with PD and DLB (Krohn et al., 2022). On one hand, variants in GBA (Honeycutt et al., 2019),

transmembrane protein 175 (TMEM175)[4] locus (Krohn, Öztürk, et al., 2020) and the 5' region of SNCA locus (Krohn, Wu, et al., 2020) represent strong risk factors for iRBD and its phenoconversion into an overt neurodegenerative disease. On the other hand, no relation has been found with other important risk variants for PD and DLB, such as LRRK2, bridging integrator 1 (BIN1), MAPT and Apolipoprotein E (APOE) (Krohn, Ruskey, et al., 2020; J. Li et al., 2018). Notably, a recent genome-wide association study (GWAS) published by Krohn and colleagues in 2022 (Krohn et al., 2022) confirmed these results, showing six RBD-associated loci in five genomic regions, i.e., SNCA, GBA, TMEM175, scavenger receptor class B member 2 (SCARB2) and inositol polyphosphate-5-phosphatase F (INPP5F). Moreover, they also found no associations with the above mentioned LRRK2, BIN1, MAPT and APOE variants (Krohn et al., 2022).

1.2.2. iRBD Pathophysiology

Sleep can be described as a state of reduced physical and mental activity comprising two main states: NREM and REM sleep, that alternate cyclically across a sleep episode (Thomas & Delphine, 2023). Specifically, REM sleep is marked by low-amplitude, mixed-frequency EEG (θ range: 4-8 Hz and α range: 8-12 Hz), muscle atonia and episodic bursts of REM (Berry et al., 2012; Kryger et al., 2017). For the generation of REM sleep, the brainstem, particularly the pons and adjacent regions of the lower midbrain, plays a crucial role (Kryger et al., 2017). As described in detail, during Braak stages I and II the brainstem is highly affected by α-syn pathology which may result in pathological changes contributing to the generation of RSWA and iRBD (see paragraph 1.1.1.). On this basis, several animal studies tried to clarify the pathophysiological association between iRBD and synucleinopathies. Studies on cats led to the conclusion that two motor systems contributed to normal REM sleep and both seemed to be impaired in iRBD, namely i) the motor system generating muscle atonia; and ii) the motor system suppressing locomotor activity (Boeve, 2010). Schenck and collaborators (Schenck & Mahowald, 2002) observed that the loss of the physiological atonia alone was not sufficient to induce RBD, but the disinhibition of locomotor activity generators was also necessary. Despite these

[4] Transmembrane protein 175 (TMEM175): is a protein-coding gene that encodes a lysosomal K^+ channel that regulates lysosomal function (Cang et al., 2015; Krohn, Öztürk, et al., 2020). For this reason, TMEM175 is crucial for the proper functioning of the ALP which is profoundly influenced by pathological changes in α-syn aggregation (as described in paragraph 1.1.1.).

findings several questions remained, particularly regarding the primary nuclei regulating REM sleep (Boeve, 2010; Boeve et al., 2007). In this context, Lu and collaborators (Lu et al., 2006) suggested a new model for REM sleep regulation in the rat. This model stated the existence of a flip-flop switch within the brainstem, involving reciprocal inhibitory interactions between two populations of GABAergic neurons: REM-off and REM-on types (Lu et al., 2006). On one hand, REM-off neurons are responsible for REM sleep deactivation, these populations are located in the ventrolateral part of the periaqueductal gray matter (vlPAG) and in the lateral pontine tegmentum (LPT). On the other hand, REM-on neurons are responsible for REM sleep activation, these populations are located in the sublaterodorsal nucleus (SLD) and in the pre-coeruleus (PC). Notably, these REM-on regions also contain glutamatergic neurons projecting from the SLD to GABAergic and glycinergic interneurons in the anterior horn of the spinal cord. These glutamatergic projections inhibit spinal motoneurons, yielding to the physiological atonia during REM sleep (Boeve et al., 2007; Lu et al., 2006). Lesion studies in the rat observed that damaging these REM-on glutamatergic neurons in the SLD cause disinhibition of spinal motoneurons, leading to presence of RSWA (Boeve et al., 2007; Lu et al., 2006). Based on evidence from animal models, it has been hypothesized that the systems and mechanisms responsible for REM sleep generation, RSWA, and iRBD may be similar to those in humans (Boeve, 2010; Boeve et al., 2007). Specifically, Boeve and colleagues (Boeve, 2010; Boeve et al., 2007) stated that the physiological inhibition of muscle activity during REM sleep could occur via two main pathways: i) a direct route projecting from the SLD (human counterpart) to the spinal interneurons; and ii) an indirect route projecting from the SLD, through the medullary magnocellular reticular formation (MCRF), to the spinal interneurons (Boeve, 2010; Boeve et al., 2007). The primary hypothesis underlying iRBD suggests that damage to the SLD could lead to diminished activation of the MCRF, resulting in less inhibition of spinal motor neurons and potentially causing RSWA. However, although damage or degeneration of the MCRF is enough to induce RSWA, it is still uncertain whether these factors alone are sufficient to trigger iRBD in humans (Boeve, 2010; Boeve et al., 2007).

1.2.3. Diagnosis and Clinical Features of iRBD

The third edition of the International Classification of Sleep Disorders (ICSD)

(American Academy of Sleep Medicine, 2014) delineated the following criteria for iRBD diagnosis:

1. Recurrent events of sleep related vocalization and/or complex motor behaviors.
2. These events must occur during REM sleep, as demonstrated by PSG.
3. PSG proves the presence of RSWA.
4. The disease is not explained by other medical conditions, drugs or substance intake.

These criteria highlight the fact that a definitive diagnosis of iRBD can only be achieved through PSG which is a system composed of different instruments: EEG, EMG, electrooculography (EOG), electrocardiography (ECG), airflow, respiratory-effort channels, oxygen saturation and videography. This system allows the detection of both abnormal behaviors during REM sleep and RSWA (Frauscher et al., 2012; Hu, 2020). Indeed, the triad of symptoms that defines the disorder comprises: abnormal vocalizations, abnormal behaviors and altered dream mentation (Boeve, 2010). Abnormal vocalizations are defined as loud and intense sounds that typically reflect unpleasant or distressing dreams. These vocalizations commonly consist in mumbling, talking, shouting and swearing; however, they can also manifest as laughing, crying, singing, whistling, or even speaking in foreign languages (Arnulf, 2012; Boeve, 2010). Moving on to abnormal behaviors, in patients with iRBD motor activity during sleep usually begins with repetitive jerks or simple movements, which then escalate into more goal-directed actions such as punching, flailing, running, or even jumping out of bed (Boeve, 2010). Notably, these violent behaviors actions can result in injuries to both the patient and their bedpartners, which is one of the main reasons that drives the patient to seek clinical help. In addition to these aggressive behaviors, iRBD patients may also exhibit a variety of complex, nonviolent actions, such as eating, smoking, dancing, or even engaging in sexual behaviors, all while remaining in bed (Arnulf, 2012; Boeve, 2010; Oudiette et al., 2009). Indeed, as postural control is compromised during REM sleep, iRBD patients cannot walk while enacting their dreams, unlike those with NREM parasomnias (Hu, 2020). Abnormal behaviors are often forceful, violent, and associated with vivid, unpleasant dreams involving fighting or fleeing from danger (Hu, 2020). Indeed, iRBD is also characterized by an altered dream mentation which usually consists

in vivid nightmares, involving scenarios where they or their loved ones are pursued or attacked by animals or people. Typically, the patient assumes the role of a defender rather than an aggressor (Boeve, 2010). If not awakened during the episode, the individual generally does not remember the dream, but if roused during an iRBD episode, they quickly regain full consciousness and can vividly recall the dream's details (Hu, 2020). In this context, Fasiello and collaborators (Fasiello et al., 2022) recently published a narrative review aiming to describe dream activity in iRBD. The authors reported that while retrospective studies in the literature suggest that dreams in iRBD are notably aggressive and violent, potentially linking these features to neurodegenerative disease progression, prospective studies contradict this by showing similar dream activities across iRBD and sRBD, as well as other parasomnias (Fasiello et al., 2022). These inconsistencies might be due to a "recall bias" where the vividness of dreams might overshadow the actual connection between dream content and iRBD (Fasiello et al., 2022).

1.2.4. From iRBD to an overt neurodegenerative disease

As described in detail, the α-synucleinopathies spectrum evolves through different stages, from the prodromal phase of iRBD and extending through overt neurogenerative disorders, mainly PD, PDD and DLB (Calabresi et al., 2023). The term prodromal refers to a period during which early indicators and symptoms of neurodegeneration appear, yet they are not pronounced enough to diagnose the full-blown disease (Berg et al., 2015). In this context, the MDS task force (Berg et al., 2015) proposed research standards for prodromal PD diagnosis which included, among clinical non-motor markers, the presence of (definitive or probable) iRBD. Indeed, it is now well-established that iRBD is not merely a parasomnia, but rather an early manifestation of an α-synucleinopathy in its initial stage (Högl et al., 2018). Given these premises, prior research estimated the risk of phenoconversion to an overt neurodegenerative disorder in iRBD (Galbiati et al., 2019; Iranzo et al., 2014; Schenck et al., 2013). In 2019, Galbiati and collaborators published a meta-analysis (Galbiati et al., 2019) aiming to quantitatively assess the risk of conversion in iRBD by considering all longitudinal studies published so far. The study revealed that the computed average rate of phenoconversion in iRBD patients was 31.95%, based on an average follow-up period of 4.75 years (± 2.43 years) (Galbiati et al., 2019). In more

detail, the likelihood of progressing from iRBD to an overt neurodegenerative disease was 33.5% within five years, 82.4% after 10.5 years, and 97% after 14.2 years of follow-up with a significantly increased risk of conversion to PD with respect to other conditions (Galbiati et al., 2019). Indeed, out of 3262 patients, 44% developed PD, 25% DLB, 7% unspecified forms of dementia, 5% MSA, 3% AD and 0.5% other forms of neurodegenerative disorders (Galbiati et al., 2019).

These findings, together with the underlying pathophysiological mechanisms and the clinical characteristics already described, highlight the importance of iRBD as an indicator for the future onset of primarily PD, but also PDD and DLB. For patients and their loved ones, this is like living with a Damocles sword hanging over their heads: the disease exhibits an extremely high conversion rate, yet the precise timing, form, or certainty of its eventual phenoconversion remains indeterminate. For this reason, the identification of timely biomarkers of conversions in iRBD patients represents a research priority (Ferini-Strambi et al., 2019; Miglis et al., 2021). Biomarkers are defined as attributes that are quantitatively measured and assessed to reflect physiological processes, pathological changes, or treatment outcomes (Marek et al., 2008). The principal uses of biomarkers encompass diagnosis, prognosis, monitoring, therapy-responsiveness or combined use (Michell et al., 2004; Miglis et al., 2021). In the context of iRBD, diagnostic biomarkers are useful not only for the assessment of phenoconversion, but also for the differential diagnosis between α-synucleinopathies (Miglis et al., 2021). Prognostic biomarkers can be employed in order to predict the rate of conversion and the severity of the disease (Miglis et al., 2021). Moreover, monitoring or therapy-responsiveness biomarkers can be applied for checking disease progression over time, but also for evaluating therapies' efficacy (Miglis et al., 2021). Indeed, iRBD offers a unique early-intervention window to test potential neuroprotective therapies before the neurodegenerative process is too advanced (Ferini-Strambi et al., 2019). Finally, combining multiple uses may be helpful for improving and advancing the diagnostic, prognostic, and surveillance functions of individual biomarkers (Miglis et al., 2021).

In the literature, different biomarkers have been identified (Ferini-Strambi et al., 2019; Miglis et al., 2021). Given the aim of the present thesis (see chapter 2), an overview of the main neuroimaging, electrophysiological, autonomic and cognitive markers for iRBD phenoconversion will be discussed in the next paragraphs.

1.2.4.1. Neuroimaging biomarkers

Neuroimaging techniques represent a key tool for investigating in vivo structural and functional brain alterations in pathological conditions, facilitating the recognition of disease-specific patterns (Bourgouin et al., 2019; Saeed et al., 2017). These unique patterns can serve as reliable biomarkers of phenoconversion, providing insights into the neuroanatomical and pathophysiological underpinnings of the disease, enhancing the accuracy of diagnoses and the prediction of the clinical trajectories of iRBD patients (Bourgouin et al., 2019; Saeed et al., 2017). In 2019, Ferini-Strambi and collaborators (Ferini-Strambi et al., 2019) published a narrative review gathering the latest evidence on biomarkers in iRBD. Notably, to further elucidate the relationship between iRBD and α-synucleinopathies, the authors illustrated the different neuroimaging biomarkers of phenoconversion following Braak staging hypothesis: i) neuroimaging biomarkers assessing extranigral damage; ii) neuroimaging biomarkers assessing nigral damage; and iii) neuroimaging biomarkers assessing cortical damage (Ferini-Strambi et al., 2019). Below, the main neuroimaging biomarkers of interest for the present thesis will be presented following the same concept.

Concerning neuroimaging biomarkers assessing extranigral damage, prior research explored the possible role of ^{123}I-MIBG-SPECT in iRBD (Bourgouin et al., 2019; Ferini-Strambi et al., 2019; Kashihara et al., 2010; Knudsen et al., 2018; Miyamoto et al., 2006, 2008). iRBD patients had a significant diminishment in cardiac ^{123}I-MIBG uptake, with reduced early and late heart-to-mediastinum (H/M) ratios, as well as reduced washout rate (WR)[5] compared to healthy controls (HC) (Kashihara et al., 2010; Knudsen et al., 2018; Miyamoto et al., 2008), but also compared to MSA patients (Miyamoto et al., 2008). Notably, iRBD patients showed a similar reduction as that found in PD patients (Knudsen et al., 2018; Miyamoto et al., 2006, 2008). Indeed, several studies demonstrated that central and preganglionic neurons are primarily impacted in MSA, whereas postganglionic neurons tend to be preserved unlike in PD (Kashihara & Yamamoto, 2006; Yoshita, 2021). As a result, cardiac ^{123}I-MIBG uptake typically remains unaffected in

[5] Early and late heart-to-mediastinum (H/M) ratio: early H/M ratio reflects the integrity and distribution of pre-synaptic nerve terminals; late H/M ratio reflects noradrenaline (NA) uptake-1 pathway function (Marzullo & Mariani, 2016).
Washout rate (WR): represents an index of the degree of sympathetic drive (Marzullo & Mariani, 2016).

MSA. For this reason, ^{123}I-MIBG uptake is considered a sensitive tool for the differential diagnosis between iRBD/PD and MSA, regardless the degree of autonomic dysfunction (Kashihara & Yamamoto, 2006; Yoshita, 2021). Notably, a recent retrospective study published in 2024 by Miyamoto T. and Miyamoto M. (Miyamoto & Miyamoto, 2024) analyzed the data of 306 PSG-confirmed iRBD patients which underwent ^{123}I-MIBG-SPECT. Out of 306 iRBD patients, 33 subjects underwent repeated ^{123}I-MIBG-SPECT showing a gradual decline in uptake over the following 4.2 years (Miyamoto & Miyamoto, 2024). Interestingly, iRBD patients with a reduced ^{123}I-MIBG uptake were more likely to convert to PD or DLB (90%); meanwhile, patients with a normal ^{123}I-MIBG uptake were more likely to progress to MSA (Miyamoto & Miyamoto, 2024).

Concerning neuroimaging biomarkers assessing nigral damage, since nigrostriatal DA degeneration represents a hallmark of α-synucleinopathies and it is considered the main cause of PD motor symptoms, several studies investigated the role of DAT-SPECT/PET in iRBD (Arnaldi et al., 2015; Bourgouin et al., 2019; Ferini-Strambi et al., 2019; Iranzo et al., 2017; Knudsen et al., 2018; Stokholm et al., 2017). These studies revealed that striatal presynaptic DA function was diminished in iRBD patients with respect to HC, though it was typically better preserved than in PD patients (Arnaldi et al., 2015; Bourgouin et al., 2019; Knudsen et al., 2018; Stokholm et al., 2017). Moreover, they also reported that the reduction in DA binding was more pronounced in the putamen than the caudate nucleus, consistent with what is seen in PD (Bourgouin et al., 2019; Knudsen et al., 2018; Stokholm et al., 2017). Of note, in 2017 Iranzo and colleagues (Iranzo et al., 2017) published a longitudinal study demonstrating that iRBD patients showed progressive alterations in DAT-SPECT over time. Specifically, the authors reported that those iRBD patients with reduced DA binding at baseline were more likely to convert to an overt neurodegenerative disease in the short term (Iranzo et al., 2017).

Finally, concerning neuroimaging biomarkers assessing cortical damage, several studies investigated possible alterations in brain metabolism using [^{18}F]FDG-PET in iRBD (Arnaldi et al., 2019; Bourgouin et al., 2019; Ferini-Strambi et al., 2019; Ge et al., 2015; Meles et al., 2018; Wu et al., 2014). Indeed, using spatial covariance analysis, abnormal metabolic brain networks have been revealed in PD, allowing the classification of PD patients based on individual metabolic patterns (Ma et al., 2007; Teune et al., 2014). In more detail, a reproducible PD-related covariance pattern (PDRP) has been

determined, marked by heightened metabolic activity in the pons, cerebellum and pallido-thalamic regions, and diminished activity in the lateral premotor and parietal association regions (Ma et al., 2007). Researchers observed that iRBD patients expressed a similar abnormal metabolic pattern, which overlapped significantly with the PDRP, the so-called RBD-related covariance pattern (RBDRP) (Bourgouin et al., 2019; Meles et al., 2018; Wu et al., 2014). However, two distinct RBDRP were identified across studies, each differently correlating with clinical features (Meles et al., 2018; Wu et al., 2014). The first network, identified by Wu and collaborators (Wu et al., 2014), included heightened metabolic activity in the pons, thalamus, frontal and sensorimotor areas, hippocampus, supramarginal and inferior temporal gyri, and cerebellum, and diminished activity in occipital and temporal brain areas. This first RBDRP strongly correlated with the severity of parkinsonism (Bourgouin et al., 2019; Wu et al., 2014). The second network, identified by Meles and colleagues (Meles et al., 2018), was characterized by increased metabolic activity in the cerebellum, brainstem, thalamus, sensorimotor cortex and hippocampus, and by decreased metabolic activity in the middle cingulate, temporal, occipital, and parietal areas. This second RBDRP showed a higher association with cognitive functioning (Bourgouin et al., 2019; Meles et al., 2018). These findings highlight the heterogeneity of iRBD clinical presentation and progression, which may reflect differences in metabolic patterns representing reliable biomarkers to quantify prodromal features of conversion in iRBD (Bourgouin et al., 2019). Notably, a recent study published in 2020 by Carli and collaborators (Carli et al., 2020) applied a metabolic connectivity approach on [^{18}F]FDG-PET data in order to explore network vulnerabilities within the α-synucleinopathies spectrum. On one hand, the nigro-striato-cortical dopaminergic network was greatly impacted in PD and DLB and it showed a substantial preservation in iRBD, in line with the absence of extrapyramidal signs in the iRBD sample (Carli et al., 2020). On the other hand, the noradrenergic network showed widespread connectivity reconfiguration in iRBD, PD and DLB (Carli et al., 2020). This is in line with the caudo-rostral spreading of α-syn pathology from the LC onwards, which is affected in the earliest stages of these diseases (Carli et al., 2020). Finally, the cholinergic network exhibited broad connectivity modifications in the whole spectrum, with PD showing mild reconfiguration and with DLB presenting extensive connectivity disruptions (Carli et al., 2020). Specifically, both DLB and iRBD showed significant

alterations in cholinergic projections to the thalamus, striatum and globus pallidus, which were instead preserved in PD (Carli et al., 2020). Indeed, different studies reported cholinergic deficits not only in DLB (Delli Pizzi et al., 2015; Mazère et al., 2017), but also in iRBD patients (Bedard et al., 2019; Stokholm et al., 2020). This network-specific vulnerability shared by DLB and iRBD may not only reflect a common pathological substrate, but also a specific biomarker for a more severe conversion phenotype in iRBD characterized by the presence of dementia (Carli et al., 2020).

1.2.4.2. Electrophysiological biomarkers

Since PSG is mandatory for a definitive diagnosis of iRBD, several studies focused on the identification of EEG biomarkers of phenoconversion by examining brain's electrical activity during sleep in iRBD patients (Ferini-Strambi et al., 2019). Indeed, interest in the interplay between sleep disruptions and neurodegeneration is growing, with evidence suggesting that sleep problems may not only result from neurodegeneration but also contribute to its development within a mutual relationship (Gorgoni & Galbiati, 2022). Specifically, it has been demonstrated that NREM sleep plays a key role in clearing neurotoxic waste (i.e., Aβ and – potentially – α-syn) through the glymphatic system (Gorgoni & Galbiati, 2022; Nedergaard & Goldman, 2020; Xie et al., 2013); thus, NREM sleep disruptions may impede this process, contributing to neurodegenerative diseases (Cordone et al., 2019; Cordone & De Gennaro, 2020; Gorgoni & Galbiati, 2022; Mander et al., 2015). Indeed, several studies reported the role played by NREM sleep as a protective factor from neurodegeneration and cognitive impairment (Cordone et al., 2019; Ju et al., 2017; Mander et al., 2015). Alterations in NREM EEG hallmarks, like SW, sleep spindles (SS), and K-complexes (KC) have been reported in MCI and AD patients and seem to correlate with cognitive performance (De Gennaro et al., 2017; Gorgoni et al., 2024; Mander et al., 2015). Regarding KC, it is defined as a sharp wave (duration: > 0.5 s; amplitude: > 75 μV) characterized by a short positive component, followed by a slower negative component, and by a final positive peak (Cash et al., 2009; Colrain, 2005; De Gennaro et al., 2000; Gandhi & Emmady, 2023). It is considered the largest graphoelement of human sleep EEG, typically occurring during stage 2 of NREM sleep (N2) and being more prominent in brain's frontal regions (Cash et al., 2009; Colrain, 2005; De Gennaro et al., 2000; Gandhi & Emmady, 2023). De Gennaro and colleagues

(De Gennaro et al., 2017), reported that AD patients showed diminished KC density (KCd, about > 40 %) with respect to HC, additionally associated with cognitive impairment. Moreover, Gorgoni and collaborators (Gorgoni et al., 2024) recently demonstrated a reduced KCd in parietal areas in amnestic MCI (aMCI) patients which later developed AD with respect to non-progressed aMCI patients and HC. Within the field of iRBD research, the only study investigating the role of KC is the one published by Galbiati and collaborators (Galbiati et al., 2021). First, the authors studied the interplay between KCd and cognitive functioning observing not only a reduced N2 KCd in iRBD patients with MCI (iRBD+MCI) with respect to iRBD patients without MCI (iRBD-MCI), but also an association between KCd and cognitive performance in global cognition, visuospatial abilities and executive function domains (Galbiati et al., 2021). While in accordance with prior research (De Gennaro et al., 2017; Gorgoni et al., 2024), this finding holds particular importance since visuospatial abilities and executive function are between the most affected domains in iRBD patients (see paragraph 1.2.4.4.) (Leitner et al., 2024). Second, the authors used [^{18}F]FDG-PET in order to explore brain metabolic underpinnings of KC in iRBD patients. Specifically, they observed an association between reduced KCd and disrupted brain metabolic connectivity in the anterior default mode network (ADMN) in iRBD+MCI patients (Galbiati et al., 2021). This evidence is probably related to the underlying neurodegenerative process. Indeed, not only cognitive dysfunction in α-synucleinopathies is strongly associated with the progressive spreading and worsening of α-syn pathology in cortical brain areas (see paragraph 1.1.1.) (Braak et al., 2003, 2004; Goedert et al., 2013), but this pathological process may also affect KC originating in the cortex (Galbiati et al., 2021; Latreille et al., 2020). Notably, spontaneous KC are considered a forerunner of SW, another NREM sleep hallmark (De Gennaro et al., 2000). SW may serve as indicators of sleep pressure and intensity, demonstrating homeostatic regulation, local modulation, and an association with memory and neural plasticity (Gorgoni & Galbiati, 2022). SW activity (SWA) refers to the amount of slow-frequency activity (0.5-4 Hz) in the EEG during sleep (Achermann et al., 2017). SWA has been proved to be reduced in AD patients showing an association with Aβ accumulation (Cordone et al., 2019; Gorgoni & Galbiati, 2022; Mander et al., 2015); moreover, a higher accumulated power of SW has been linked to a slower motor progression in PD patients (Gorgoni & Galbiati, 2022; Schreiner et al., 2019). Given these

premises, the role of SW has also been investigated in iRBD. In this context, it has been demonstrated that iRBD patients show a reduced overnight decline in SWA (Valomon et al., 2021), reduced amplitude, longer duration and shallower slope of slow oscillations (SO)[6] (Sunwoo et al., 2021) compared to HC; however, another study observed no differences in SW features between iRBD patients and HC (Latreille et al., 2011). Finally, also SS characteristics have been explored in RBD patients. SS are another N2 sleep hallmark and are defined as rhythmic bursts of activity (11–15 Hz) beginning with a rising amplitude, peak, and then gradually diminishing (De Gennaro & Ferrara, 2003). These graphoelements arise from thalamocortical mechanisms and have been functionally linked to cognitive processes (i.e., intelligence and memory) and to brain development (De Gennaro & Ferrara, 2003; Gorgoni & Galbiati, 2022). Since disruption of SS have been observed both in AD and PD (Gorgoni et al., 2016; Gorgoni & Galbiati, 2022; Latreille et al., 2015), it has been hypothesized a possible role of SS as potential biomarkers of a malignant conversion subtype in iRBD. Indeed, studies on iRBD consistently reported SS alterations comprising reduced SS density in both iRBD and PD with RBD patients with respect to HC, specifically in centro-parietal areas, potentially linked to thalamo-cortical networks alterations due to α-syn pathology (Christensen et al., 2015; Gorgoni & Galbiati, 2022; O'Reilly et al., 2015). Overall, only a few studies explored NREM sleep characteristics in iRBD and the relationship between alterations in NREM graphoelements and phenoconversion, with conflicting results likely due to methodological differences (Gorgoni & Galbiati, 2022). Despite these limits, NREM sleep seems to play a promising role in this context, starting from its neuroprotective effects against neurodegeneration, as will be demonstrated in chapter 3.

Beyond NREM alterations, EEG slowing has also been observed during both REM sleep and wakefulness in iRBD patients (Ferini-Strambi et al., 2019). Previous studies on AD and MCI patients reported EEG slowing during REM sleep characterized by an increase in low-frequency δ activity on fronto-temporal regions and a reduction in high-frequency β activity on temporal regions, mirroring the patterns observed during wakefulness (D'Atri et al., 2021; Ferini-Strambi et al., 2019; Gorgoni & Galbiati, 2022).

[6] Slow oscillations (SO): SO are defined as < 1 Hz EEG activity reflecting repeated depolarizations (UP state) and hyperpolarizations (DOWN states) in membrane potentials of cortical neurons (Crunelli & Hughes, 2010; Steriade et al., 1993; Sunwoo et al., 2021).

This slowing, which was proved to be associated with cognitive decline, is likely linked to the impairment of the cholinergic system and may serve as a potential marker of progression also in iRBD, as cholinergic deficits have also been reported in these patients (see paragraph 1.2.4.1.) (Bedard et al., 2019; Carli et al., 2020; Stokholm et al., 2020; Gorgoni & Galbiati, 2022). During wakefulness, it has been demonstrated that iRBD patients who later converted showed EEG slowing characterized by increased absolute power in δ and θ bands across frontal, central, parietal, temporal, and occipital brain areas, compared to stable iRBD patients and HC (Brazète et al., 2016). Similar findings were reported by Fantini and collaborators (Fantini et al., 2003) by observing not only increased power in θ band on frontal, temporal, and occipital regions, but also with reduced power in β band on occipital areas during wakefulness in iRBD patients compared to HC. Moreover, the authors also reported reduced power in β band on occipital regions (Fantini et al., 2003). In agreement with these results and with previous findings on MCI and AD patients, Iranzo and collaborators (Iranzo et al., 2010) reported pronounced EEG slowing in iRBD+MCI characterized by increased δ and θ bands activity on central and occipital brain areas during both wakefulness and REM sleep, compared to iRBD-MCI. Notably, EEG spectral profile showed by iRBD+MCI resembled that observed in PDD/DLB patients, supporting the possible role of abnormal EEG activity as a potential biomarker of phenoconversion to a more malignant conversion subtype in iRBD patients (Ferini-Strambi et al., 2019; Iranzo et al., 2010).

1.2.4.3. Autonomic markers

As previously outlined, α-synucleinopathies are linked to a broad spectrum of non-motor manifestations, with dysautonomia considered a hallmark of these disorders, often preceding the occurrence of both motor and cognitive features by many years (Bae et al., 2009; Chiaro et al., 2018; Hu et al., 2022; Inoue & Sasai-Sakuma, 2019; Oka et al., 2020; Poewe, 2007; Poewe et al., 2017; Thaisetthawatkul et al., 2004). On one hand, severe autonomic dysfunction is a supportive clinical feature for DLB diagnosis (McKeith et al., 2017). On the other hand, it is also a common non-motor symptom of PD, related to cognitive decline and PDD (Kim et al., 2009; Poewe, 2007). This mirrors α-syn pathology spreading, with brainstem regions neurodegeneration (i.e., LC and dorsal motor nucleus of the CN X) not only contributing to RSWA and iRBD (see paragraph 1.2.2.), but also

playing a key role in autonomic dysfunction (Braak et al., 2003, 2004; Inoue & Sasai-Sakuma, 2019). Hence, autonomic nervous system (ANS) abnormalities in RBD patients might serve as markers of phenoconversion (Chiaro et al., 2018; Inoue & Sasai-Sakuma, 2019). In this context, several studies observed autonomic dysfunction related to several domains in iRBD patients, such as cardiovascular (i.e., OH and abnormal heart rate variability), gastrointestinal and genitourinary (i.e., constipation, urinary and erectile dysfunction), respiratory, thermoregulation and pupillomotor (Ferini-Strambi et al., 2014; Frauscher et al., 2012; Inoue & Sasai-Sakuma, 2019; Postuma et al., 2009).

Given the aims of the present thesis (see chapter 2), an overview of autonomic dysfunctions related to the pupillomotor domain will be provided below. Indeed, previous studies on PD and AD reported an altered pupillary light reflex (PLR) in these patients, suggesting the presence of cholinergic dysfunction (Inoue & Sasai-Sakuma, 2019). Indeed, the PLR is defined as the change in the size of the pupil after the presentation of a light stimulus (Larson & Behrends, 2015), and it evolves through three phases (see Figure 8): i) a fast constriction phase (after the light stimulus); ii) a fast re-dilation phase; and iii) a slow re-dilation phase, where the pupil returns to the baseline condition (Wang et al., 2016).

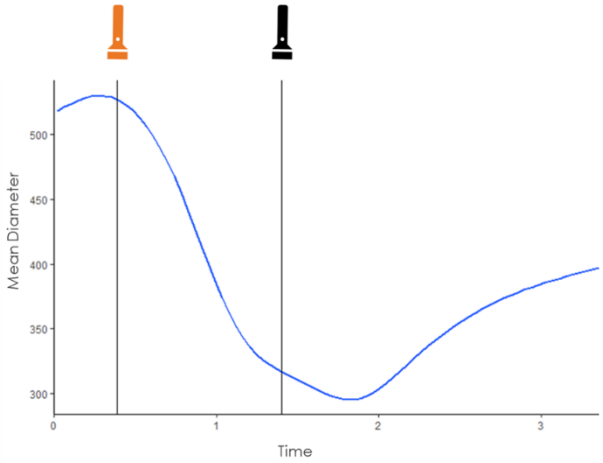


Figure 8. Graphical representation of the PLR and its phases. This figure was created by me. After the presentation of the light stimulus, the pupil undergoes a phase of fast constriction, immediately followed by a phase of fast re-dilation, finishing with a slow re-dilation where the pupil returns to the baseline condition. The orange flashlight depicts the onset of the light stimulus; the black flashlight depicts the offset of the light stimulus.

These two mechanisms, constriction and re-dilation, take place thanks to two groups

of antagonistic muscles: the iris dilator muscle, allowing pupil re-dilation (controlled by the sympathetic nervous system (SANS)), and the iris sphincter muscle, allowing pupil constriction (controlled by the parasympathetic nervous system (PANS)) (Larson & Behrends, 2015). In more detail, when a light stimulus is presented to the retina, it activates the neurons located in the Edinger-Westphal nucleus. This leads to the excitation of the preganglionic PANS neurons innervating the ciliary ganglion and to the subsequent activation of the iris sphincter muscle, which allows pupil constriction to take place (Wang et al., 2016). After this first phase, the pupil undergoes re-dilation through two coordinated mechanisms controlled by the SANS: a) inhibition of PANS neurons innervating the iris sphincter muscle, resulting in muscle relaxation and subsequent pupil enlargement; and b) stimulation of the peripheral sympathetic nerve, which allows the contraction of the iris dilator muscle (Hall & Chilcott, 2018). Notably, the iris sphincter muscle and its PANS projections are mainly driven by the cholinergic system, with acetylcholine (ACh) being its main neurotransmitter. Thus, cholinergic dysfunctions, often seen in α-synucleinopathies (see paragraphs 1.1.3. and 1.2.4.1.), might result in an altered PLR in these conditions, serving as a peripheric marker of cholinergic deficiency (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009). Since the pupillometric exam is considered a fast, low-cost, non-invasive method allowing the objective evaluation of the PLR and its parameters (Larson & Behrends, 2015), several studies investigated cholinergic system functioning in neurodegenerative diseases using this technique (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009). Indeed, changes in PLR parameters may reflect the underlying presence of cholinergic impairment resulting in a reduced PANS activity (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009) and the presence of cognitive decline (Bedard et al., 2019; Carli et al., 2020; Delli Pizzi et al., 2015; Gersel Stokholm et al., 2020; Kehagia et al., 2012; Mazère et al., 2017). The main parameters extracted from PLR analysis include: the baseline pupil diameter, referring to pupil size after dark adaptation (before the presentation of the light stimulus); the variation in pupil diameter, define as the change in pupil size after the presentation of the light stimulus; the latency of the pupil reaction to the light stimulus; the maximum constriction velocity (VCmax) of the pupil; the maximum constriction acceleration (ACmax) of the pupil (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009). In this context, Frost and colleagues (Frost et al., 2017) compared the PLR between

14 patients with AD and 115 HC, the latter stratified based on Aβ burden. The authors not only observed an altered PLR in AD patients compared to HC (i.e., reduced VCmax and ACmax), but also that HC with a high Aβ burden showed an altered PLR and a greater cognitive decline with respect to HC with low Aβ burden (Frost et al., 2017). In addition, Fotiou and colleagues (Fotiou et al., 2009) compared the PLR of 23 HC to 23 probable AD, 11 PD+MCI and 11 PD-MCI patients. The results showed an altered PLR in probable AD patients, PD+MCI and PD-MCI patients with respect to HC. Moreover, PD+MCI also presented reduced VCmax and ACmax compared to PD-MCI (Fotiou et al., 2009). Notably, while ACmax was the most accurate indicator for distinguishing between HC and probable AD individuals, both ACmax and VCmax were able to accurately classify a subject as HC, PD-MCI or PD+MCI (Fotiou et al., 2009). In agreement with these data, Stergiou and colleagues (Stergiou et al., 2009) compared the PLR of 11 HC to 11 PD+MCI and 11 PD-MCI patients. The authors reported that PD+MCI showed reduced VCmax and ACmax compared to both HC and PD-MCI patients, representing the most sensitive classifiers (Stergiou et al., 2009). Overall, these results support the presence of an underlying cholinergic impairment both in AD and PD patients, which seems to be mainly related to cognitive impairment in both populations. Moreover, they also support the role of pupillometry in the early peripherical detection of cholinergic deficiency, with VCmax and ACmax being its most sensitive indicators (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009). As outlined above, both cholinergic and autonomic dysfunctions have also been reported in iRBD patients, representing major risk factors for neurodegeneration (Carli et al., 2020; Ferini-Strambi, Oertel, et al., 2014; Stokholm et al., 2020; Inoue & Sasai-Sakuma, 2019). In this context, a few recent studies studied pupillary functioning in iRBD (La Morgia et al., 2022; Perkins et al., 2021; Sunwoo et al., 2022). However, two of these studies respectively used a video-based monocular eye-tracker (Perkins et al., 2021) and a chromatic pupillometry (La Morgia et al., 2022), which provide different measures of pupil size and pupillary reflexes. Thus, to the best of current knowledge, only the study of Sunwoo and colleagues (Sunwoo et al., 2022) assessed PLR functioning in iRBD using a portable pupillometer (similar to previous studies on AD and PD) on 27 iRBD patients and 23 PD patients. The results showed that, despite differences in minimum and maximum pupil diameters, all the other PLR parameters (VCmax, ACmax, latency, variation) did not differ between iRBD and PD patients suggesting a

similar degree of pupillary autonomic dysfunction in these two conditions (Sunwoo et al., 2022). In summary, these findings collectively emphasize the presence of autonomic dysfunction across the entire spectrum of α-synucleinopathies, from the earliest prodromal stage (iRBD) to full-blown disorders (PD) (Fotiou et al., 2009; Frost et al., 2017; Stergiou et al., 2009; Sunwoo et al., 2022). Specifically, existing research suggests not only that PLR dysfunction is already present in iRBD patients (Sunwoo et al., 2022), but it may also play a crucial role as an autonomic marker of conversion mainly linked to cognitive decline. However, since the paucity of studies in the literature, further research is needed to assess cholinergic deficiency in iRBD patients using pupillometry, as well as to explore the relationship between PLR abnormalities and the risk of phenoconversion in this population.

1.2.4.4. Cognitive markers

Prior research showed that most iRBD patients exhibit and develop cognitive deficits, showing a progressive decline (Fantini et al., 2011; Marchand et al., 2018). Moreover, iRBD neuropsychological profile is characterized by cognitive dysfunctions similar to that found in patients with an overt α-synucleinopathy (see paragraph 1.1.2.) (Leitner et al., 2024). For these reasons, the role of iRBD cognitive profile has been extensively investigated as a prodromal marker of phenoconversion, proving highly valuable in identifying patients' clinical trajectories (Arnaldi et al., 2021; Ferini-Strambi et al., 2014; Marchand et al., 2018; Marchand et al., 2017; Terzaghi et al., 2008). However, there is considerable variability in the literature regarding the cognitive domains most affected in iRBD (Ferini–Strambi et al., 2004; Leitner et al., 2024; Marchand et al., 2017, 2018; Massicotte-Marquez et al., 2008). Most cross-sectional studies reported that these patients exhibit deficits mainly in memory and executive function domains (Massicotte-Marquez et al., 2008; Rolinski, Griffanti, et al., 2016; Rolinski, Zokaei, et al., 2016). However, other cross-sectional studies highlighted impairments in visuospatial abilities (Fantini et al., 2011; Ferini–Strambi et al., 2004). In contrast, longitudinal studies demonstrated that only deficits in the executive function domain seemed to forecast the progression from iRBD to an overt neurodegenerative disorder (Marchand et al., 2017, 2018; Youn et al., 2016). Among these studies, Marchand and colleagues (Marchand et al., 2017) published a prospective study including 76 iRBD patients which underwent a complete

neuropsychological assessment. Thirty-four of these patients (45%) converted to an overt neurodegenerative disease after 3.6 years: 19 patients first developed parkinsonism, and 15 patients first developed dementia (Marchand et al., 2017). At baseline, iRBD patients who developed a dementia-first form not only had a higher prevalence of MCI (93%), but they also showed deficits across all cognitive domains i.e., attention, executive function, visuospatial abilities and memory (Marchand et al., 2017). Conversely, those who developed a parkinsonism-first form displayed a cognitive profile similar to iRBD patients who remained stable (Marchand et al., 2017). The following year, Marchand and colleagues (Marchand et al., 2018) published another study describing cognitive changes occurring in 109 iRBD patients over a prodromal period of three to six years. At baseline, iRBD patients who developed DLB already exhibited significant impairments in attention and executive function domains up to six years before diagnosis (Marchand et al., 2018). Although memory and episodic verbal learning were also reduced up to five to six years prior to DLB diagnosis, these deficits only became of medical significance one to two years before conversion (Marchand et al., 2018). In contrast, iRBD patients who developed PD showed reduced performance in attention and executive function domains only a few years prior to PD diagnosis (Marchand et al., 2018). These findings support the hypothesis that iRBD patients may show different cognitive profiles based on their future phenoconversion (Marchand et al., 2018). In order to overcome the heterogeneity of the literature, we recently published a meta-analysis (Leitner et al., 2024) with two primary aims: 1) to compare the cognitive profile of iRBD patients to that of HC; and 2) to quantitatively estimate the risk of developing a neurodegenerative disease in iRBD patients based on cognitive deficits. In line with the first aim, the meta-analysis of cross-sectional studies demonstrated that iRBD patients, compared to HC, showed the greatest impairments in cognitive screening, memory, and executive function domains, with only reduced performance in language and visuospatial abilities (Leitner et al., 2024). In line with the second aim, the meta-analysis of longitudinal studies revealed greater baseline impairments in executive function among patients who converted to an overt neurodegenerative disorder with respect to those who remained stable over time (Leitner et al., 2024). These results align with previous research suggesting the predictive role of executive function domain as a marker of progression in iRBD (Marchand et al., 2017, 2018; Youn et al., 2016).

In addition to cognitive profile, several studies observed that MCI in iRBD may serve as a marker of conversion (Arnaldi et al., 2021; Marchand et al., 2017, 2018; Massicotte-Marquez et al., 2008; Postuma et al., 2019; Rahayel et al., 2021; Terzaghi et al., 2019). As previously mentioned, the prevalence of MCI in iRBD has been calculated to be approximately 50% (Gagnon et al., 2009; Marchand et al., 2017). Notably, the presence of MCI at baseline has been shown to heighten the risk of phenoconversion threefold in iRBD (Leitner et al., 2024; Terzaghi et al., 2019). Furthermore, longitudinal studies demonstrated that iRBD+MCI patients develop dementia more quickly than iRBD-MCI (Arnaldi et al., 2021; Marchand et al., 2017; Postuma et al., 2019; Terzaghi et al., 2013). Indeed, the presence of MCI in iRBD has been specifically linked to a more malignant phenotype, characterized by pronounced DA neurodegeneration in the nigrostriatal pathway and frequent occurrence of constipation (Arnaldi et al., 2021), as well as an increased risk of dementia (i.e., PDD/DLB) (Arnaldi et al., 2021; Marchand et al., 2017, 2018; Massicotte-Marquez et al., 2008; Postuma et al., 2019; Rahayel et al., 2021; Terzaghi et al., 2019). In this context, a few studies characterized the MCI profile within the iRBD population based on the nature of the impairment (i.e., aMCI, non-amnestic MCI (naMCI)), the number of affected cognitive domains (i.e., MCI single-domain (MCIsd), MCI multi-domain (MCImd)), and the MCI subtype (i.e., amnestic MCI single-domain (aMCIsd), amnestic MCI multi-domain (aMCImd), non-amnestic MCI single-domain (naMCIsd), non-amnestic MCI multi-domain (naMCImd)) (Gagnon et al., 2009; Mombelli et al., 2023). Gagnon and colleagues (Gagnon et al., 2009) reported that iRBD patients more frequently presented naMCIsd, primarily characterized by impairments in executive function and attention. In a recently published article (Mombelli et al., 2023), we identified three latent classes of iRBD based on neuropsychological test scores, characterized by slight, moderate, or severe cognitive impairment (Mombelli et al., 2023). The "severely impaired" group, which exhibited deficits in all cognitive domains, had the highest prevalence of MCI (80.7%), specifically aMCImd (64.2%) followed by naMCImd (17.9%) (Mombelli et al., 2023). In contrast, the "moderately impaired" group had a MCI prevalence of 59.1% and the "slightly impaired" group of 14.8%, with 50% of the latter showing aMCIsd (Mombelli et al., 2023). In summary, this evidence highlights the importance of examining both the neuropsychological profile and the presence of MCI in iRBD, as they may serve as important predictive markers of phenoconversion.

2. AIM OF THE WORK

Since iRBD is recognized worldwide as a prodromal stage of α-synucleinopathies, the identification of biomarkers for a timely prediction of phenoconversion is a research priority in this field. The main aim of the present thesis is to explore the role of behavioral, neuropsychological and neurophysiological measures, as possible early markers for disease progression in iRBD patients.

In more detail, this articulates in three specific aims:

1. Exploring the role of cognitive reserve (CR) in iRBD in two ways. First, by evaluating the impact of CR on the cognitive functioning of iRBD patients. Second, by investigating the relationship between SW and PDRP, alongside cognitive performance in iRBD, with an exploratory focus on CR.
2. Investigating the possible presence of early peripheric cholinergic dysfunction in iRBD patients by means of pupillometry and the evaluation of the PLR. Moreover, testing whether this dysfunction mediates the presence of cognitive impairment in iRBD patients.
3. Evaluating whether temporal processing and sensorimotor synchronization dysfunctions may be already present in iRBD patients and to investigate resting-state EEG-related rhythms.

Collectively, these aspects will help construct a comprehensive understanding of the dysfunctions underlying iRBD patients. Beyond deepening our understanding of the neurobiological mechanisms involved, they provide critical insights into the identification of clinical trajectories within the α-synucleinopathies spectrum.

3. COGNITION, NEUROIMAGING & NEUROPHYSIOLOGY: COGNITIVE AND BRAIN RESERVE IN iRBD

Data of Study I are already published (D'Este et al., 2023).

3.1. Study I. Cognitive Reserve in iRBD

As previously described, iRBD is recognized worldwide as a prodromal stage of α-synucleinopathies such as PD and DLB (Galbiati et al., 2019; Postuma et al., 2019). It is quite established that after 15–20 years of disease duration, the vast majority of iRBD patients (> 90 %) develop a full-blown α-synucleinopathy (Galbiati et al., 2019). However, the time of progression is highly heterogeneous among these patients. The median time of progression varies between 12 to 14 years, but a considerable portion of iRBD patients (~33.5 %) shows a faster conversion – five years of disease duration (Galbiati et al., 2019). The reason underlying this inter-subject variability is still largely unknown. Both modifiable (e.g., life experience) and non-modifiable (e.g., genetic and sex) factors might modulate the speed of conversion rate in iRBD, delaying or accelerating motor and/or cognitive symptoms onset.

The CR might play a role in this phenomenon, contributing to the delay of the phenoconversion in iRBD. Brain reserve (BR) capacity seems to protect against pathological cognitive decline, as in the case of neurodegenerative diseases (Barulli & Stern, 2013). Specifically, individuals with more reserve might better cope with neurodegenerative processes due to an active compensatory mechanism lying on enhanced brain network efficacy and flexibility (Tucker & Stern, 2011). Life-long experiences can influence the efficacy and flexibility of brain networks by promoting neural plasticity. On this basis, education, stimulating occupational experience, social environments and leisure activities can modulate the brain architecture, contributing to building up CR. In other words, individuals with a high reserve have greater neural resources, requiring more pathology burden to reach the critical threshold needed to clinically manifest ongoing pathological mechanisms (delay in the onset of clinical symptoms).

The theoretical framework of CR has been extensively investigated both in the context of normal ageing (Tucker & Stern, 2011; Stern, 2012) and in neurodegenerative diseases

(Stern, 2012; Xu et al., 2015). Regarding PD, the literature reports that education acts as a protective factor in PD patients (Cubo et al., 2002; Hindle et al., 2014). PD patients with higher education have better performances in different cognitive domains (i.e., attention, executive function, memory and visuospatial abilities) (Hindle et al., 2014). Few studies addressed the role of CR in DLB patients, the majority focusing on education; however, results are highly heterogeneous (Lamotte et al., 2016; Perneczky et al., 2007, 2008, 2009). One study focused on the role of occupation as a proxy of CR in DLB, showing that specific job skills (i.e., problem-solving, visual tasks and sociality) act as protective factors in DLB, shaping the connectivity of high-order neural networks (Carli et al., 2021). Although studies on reserve are particularly significant in the preclinical stage of neurodegenerative diseases, there is no evidence regarding CR in iRBD. Recently, a cross-sectional study evaluated the possible moderating action of education in subjects diagnosed with probable RBD (questionnaire-based diagnosis) (Chen et al., 2020). The authors demonstrated that a high level of education delays the onset of cognitive and motor decline in these patients (Chen et al., 2020).

The main aim of the present study was to evaluate the impact of CR on the cognitive functioning of iRBD patients. Investigating the effect of long-life experience in this prodromal condition might help to explain the inter-subject variability in the progression rate. However, quantifying CR is often a hard challenge. It is increasingly clear that sustained engagement in multiple activities critically develops the CR (Richards & Deary, 2005). Thus, focusing on static contributors (experience in a single period) makes it difficult to capture either the impact of prolonged exposure or the interplay of several reserve-enhancing factors (Fratiglioni et al., 2004; Reed et al., 2011). In 2012, Nucci and colleagues (Nucci et al., 2012) developed a standardized questionnaire (the Cognitive Reserve Index questionnaire (CRIq)) to measure CR in all its complexity to overcome this challenge. This tool takes into account different life experiences (education, occupation and leisure activities) and the time spent in each. Thus, for instance, it considers whether a subject changed jobs during the lifespan and weighs each experience according to the time duration. In addition, this instrument provides separate scores for each evaluated life aspect and a total score for the integration of all the variables. Thus, we used CRIq to quantify CR accumulated by individuals through their lifespan and to assess its impact on their cognitive functioning.

3.1.1. Methods

3.1.1.1. Participants

Fifty-five PSG-confirmed iRBD patients were retrospectively recruited between 2017 and 2022 from the clinical database of the Sleep Disorders Center, San Raffaele Hospital, Milan (Italy). The clinical diagnosis of iRBD was made by a sleep-medicine expert according to the third edition of the ICSD (American Academy of Sleep Medicine, 2014). For the present study, exclusion criteria were the presence of dementia, parkinsonism or any other psychiatric or neurological condition (American Psychiatric Association, 2013; Postuma et al., 2015). All patients underwent a comprehensive clinical evaluation, including neurological examination, video-PSG exam, neuropsychological assessment, motor and non-motor symptoms questionnaires and the CRIq. All participants provided written informed consent to the experimental procedure, which was previously approved by the local ethical committee.

3.1.1.2. Video-PSG

Video-PSG recording included 19-channel EEG placed according to the international 10–20 system (Klem, 1999): EMG of the submentalis, flexor digitorum superficialis and anterior tibialis muscles (i.e., SINBAR montage) (Frauscher, Iranzo, et al., 2012); two-channels EOG (i.e., right and left); ECG; and airflow and respiratory-effort channels. Sleep was scored by a sleep-medicine expert according to the international guidelines of the American Academy of Sleep Medicine (AASM) using 30s epochs (Berry et al., 2012).

3.1.1.3. Neuropsychological Evaluation

The standard neuropsychological protocol included a comprehensive battery assessing five global cognitive domains by means of different standardized tests: (1) the cognitive screening domain was assessed by means of the Mini-Mental State Examination (MMSE) (Measso et al., 1993); (2) the language domain was assessed by means of the token test (De Renzi & Faglioni, 1978), semantic and phonemic verbal fluency tests (Novelli et al., 1986) and the CAGI oral denomination test (Catricalà et al., 2013); (3) the memory domain was assessed by means of the digit-span test (forward (Orsini et al., 1987) and backward (Kessels et al., 2008) versions), the Corsi block-tapping test (Spinnler & Tognoni, 1987), the Rey auditory verbal learning test (RAVLT) (i.e., immediate recall,

delayed recall and recognition parts) (Carlesimo et al., 1996) and the recall of the Rey–Osterrieth complex figure (ROCF) (Caffarra et al., 2002); (4) the executive function domain was assessed by means of Raven's progressive matrices test (RPM) (Spinnler & Tognoni, 1987) and the attentive matrices test (Spinnler & Tognoni, 1987); finally, (5) the visuospatial abilities domain was assessed by means of the copy of the ROCF (Caffarra et al., 2002). The neuropsychological evaluation was performed by an expert neuropsychologist, who administered and scored each test according to national procedures. Specifically, raw scores obtained in each neuropsychological test were adjusted for age, gender and education, providing adjusted scores for each patient (corrected scores). Then, corrected scores were evaluated based on a five-level scale: (0) severe deficits (i.e., pathological threshold), (1) moderate deficits, (2) mild deficits, (3) minimal deficits, and (4) no deficits. Based on this neuropsychological evaluation, the presence of MCI was assessed, and a clinical diagnosis was made according to the criteria proposed by Petersen and colleagues in 2018 (Petersen et al., 2018).

3.1.1.4. Motor and Non-Motor Symptoms Questionnaires

Motor and non-motor symptoms were evaluated through the administration of standardized questionnaires, namely the UPDRS (Goetz et al., 2008), the Non-Motor Symptoms Questionnaire (NMSQ) (Chaudhuri et al., 2006) and the Scale for Outcomes in Parkinson's Disease – Autonomic (SCOPA-AUT) (Visser et al., 2004).

3.1.1.5. CRIq

The CRIq was administered to each patient. It represents a standardized and reliable tool for measuring CR throughout the lifespan (Nucci et al., 2012). It investigates three different CR proxies: (i) education, i.e., years of schooling and other possible training courses; (ii) working activity, i.e., the number of years for each of the professions carried out, and the type of job scored on five degrees of intellectual demand (i.e., class 1: unskilled manual work; class 2: skilled manual work; class 3: skilled non-manual or technical work; class 4: professional occupation; class 5: highly intellectual occupation); and (iii) leisure time, including the number of years, type and frequency of different cognitively stimulating activities (i.e., intellectual, social and physical activities) (Nucci et al., 2012). The CRIq returns one composite total score and three different subscores,

one for each evaluated CR proxy. Of note, the CRIq total score is classified into five different levels: level 1, low CR (less than 70); level 2, medium–low CR (70–84); level 3, medium CR (85–114); level 4, medium–high CR (115–130); and, level 5, high CR (more than 130) (Nucci et al., 2012).

3.1.1.6. Statistical Analyses

Data were analyzed using Statistical Package for Social Sciences (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.) and Jeffreys's Amazing Statistics Program (JASP, Version 0.16.0, Amsterdam, NL) software. First, we performed the descriptive statistics to characterize the sample in terms of gender, age, level of education, presence of hyposmia, disease duration, presence of MCI, presence of motor and nonmotor symptoms, and PSG indexes. Corrected neuropsychological test scores were compared among patients divided according to the CRIq level. The disease duration might be a confounding variable in assessing CR modulation of neuropsychological scores; the cognitive performance of iRBD can decrease with disease progression. This requires excluding the effect of disease duration on the tested CR models. To do this, we entered disease duration as a covariate in the group comparisons. We also wanted to investigate differences across global cognitive-domain scores (rather than across single neuropsychological test scores) among subjects grouped according to their CRIq level. We first z-transformed the corrected scores of each neuropsychological test. Then, we averaged the z-scores of the neuropsychological tests belonging to a specific cognitive domain: cognitive screening, memory, language, executive function and visuospatial abilities (see the "Neuropsychological evaluation" section for the composition of cognitive domains). This way, we obtained global z-scores for each cognitive domain. Finally, we computed the differences in the cognitive domains among CRIq level groups, controlling for disease duration. Correlation analyses were performed between the neuropsychological tests' corrected scores and CRIq values (total and subscores). In addition, we investigated the correlation between CRIq values and corrected neuropsychological scores with disease duration. The Shapiro–Wilk normality test and outlier analyses were used to assess sample distribution. All the variables were normally distributed, and the statistically significant outliers were excluded from the analyses. Comparisons of parametric variables were performed using univariate ANOVA, and

Bonferroni correction was applied for post-hoc pairwise comparisons. Pearson's rho parametric test was used in all the correlation analyses. For all the analyses, p-values less than 0.05 were considered statistically significant.

3.1.2. Results

3.1.2.1. Clinical Characteristics of the Sample

Demographic and clinical characteristics of the whole sample (n = 55) are reported in Table 3. The majority of patients were males (n = 44; 80 %) and the mean age was 66.38 ± 7.51 years. iRBD patients showed a mean disease duration of 4.26 ± 3.56 years. The disease duration was collected retrospectively, computing the time between subjective symptom onset and the time of diagnosis. Of note, the neuropsychological assessment was made at the time of the diagnosis. iRBD patients showed disturbed sleep (i.e., low percentage of sleep efficiency, high sleep latency and high wake after sleep onset), despite a good percentage of SW sleep (mean: 20.34 ± 10.04) and REM sleep (mean: 20.38 ± 6.73). The neuropsychological evaluation demonstrated that 18 patients out of 55 were affected by MCI (32.73 %), six of them had MCImd (33.33 %) and four patients presented aMCI (22.22 %). iRBD+MCI were significantly (p = 0.049) older (mean: 69.17 ± 7.29) than iRBD-MCI (mean: 65.24 ± 7.30). With respect to motor and non-motor symptoms, patients showed a mean UPDRS total score of 7.59 ± 5.59, a mean SCOPA-AUT total score of 8.66 ± 6.04 and a mean NMSQ total score of 26.00 ± 21.18; moreover, approximately half of the subjects had hyposmia (n = 26; 50.98 %).

	n = 55	%
Male	44	80.00%
Hyposmia (yes) [a]	26	50.98%
MCI	18	32.73%
	Mean ± SD	**Min–Max**
Age (years)	66.38 ± 7.51	50–78
Disease duration (years)	4.26 ± 3.56	0–17
Years of education (years)	12.38 ± 4.11	5–23
Sleep latency (min)	28.18 ± 26.49	1–123
WASO (min)	70.14 ± 43.02	13–247
TST (min)	361.18 ± 58.17	221–462
%SE	78.45 ± 10.93	41.70–94.20
NAWK	16.24 ± 12.63	5–94
N1 (%)	11.66 ± 3.92	3.80–23.40
N2 (%)	48.45 ± 8.63	29.20–69.40
Slow-wave seep (%)	20.34 ± 10.04	4–51.50
REM sleep (%)	20.38 ± 6.73	6.80–39.20
REM latency (min)	99.61 ± 55.76	13.50–265
UPDRS total score [b]	7.59 ± 5.59	0–22
SCOPA total score [c]	8.66 ± 6.04	0–26
NMSS total score [d]	26.00 ± 21.18	0–71
CRI—education	108.04 ± 13.58	84–140
CRI—working activity	111.44 ± 17.84	72–159
CRI—leisure time	110.65 ± 22.67	72–165
CRI—total	113.33 ± 18.66	77–156

Table 3. Population characteristics. Abbreviations: MCI: mild cognitive impairment; SD: standard deviation; min: minutes; WASO: wake after sleep onset; TST: total sleep time; %SE: sleep efficiency; NAWK: number of awakenings; N1: stage-one sleep; N2: stage-two sleep; REM: rapid eye movement; UPDRS: Unified Parkinson's Disease Rating Scale; SCOPA: Scales for Outcomes in Parkinson's Disease; NMSS: Non-Motor Symptoms Scale; CRI: Cognitive Reserve Index. [a] Only 51 out of 55 had hyposmia information. b Only 42 out of 55 had UPDRS total score. c Only 47 out of 55 had SCOPA total score. d Only 39 out of 55 had NMSS total score.

3.1.2.2. Characterization of the Sample According to CRIq

Regarding CR profiles, patients had a mean CRIq total score of 113.33 ± 18.66; meanwhile, the mean scores obtained in each CR proxy were as follows: education 108.04 ± 13.58, working activity 111.44 ± 17.84, and leisure time 110.65 ± 18.66 (see Table 3). Table 4 illustrates the distribution and demographic characteristics of the patients within the different levels of the CRIq. In this context, two important points should be emphasized: (i) in the present sample, none of the patients belonged to the low CR group (level 1); and (ii) the number of subjects included in CRIq level 2 group (i.e., the lowest CRIq level in the present study) was very small since only three patients fell within this category.

CRIq Level	Cut-Off	n (%)	M/F	Age (Mean ± SD)	Disease Duration (Mean ± SD)
CRIq level 2	70–84	3 (5.45%)	2/1	71.25 ± 6.40	3.67 ± 1.53
CRIq level 3	85–114	25 (44.45%)	19/6	65.77 ± 7.05	3.43 ± 2.71
CRIq level 4	115–130	17 (32.73%)	14/3	64.89 ± 8.08	4.72 ± 3.85
CRIq level 5	≥130	10 (18.18%)	9/1	69.00 ± 7.18	5.27 ± 4.76

Table 4. Characteristics of the groups based on CRIq levels at baseline. Abbreviations: CRIq: Cognitive Reserve Index questionnaire; M: males; F: females; SD: standard deviation.

3.1.2.3. MCI Distribution across CRIq Levels

In order to test whether higher CR levels may represent a possible protective factor against cognitive decline, the distribution of MCI within the groups was investigated. Table 5 illustrates how iRBD + MCI and iRBD - MCI patients were distributed within the four different groups of the CRIq. Interestingly, in the highest level of the CRIq most of the subjects with CRIq level 5 did not present MCI (9 out of 10, 90.00 %).

CRIq Level	iRBD-MCI		iRBD + MCI	
	n	%	*n*	%
CRIq level 2	2	66.67%	1	33.33%
CRIq level 3	17	68.00%	8	32.00%
CRIq level 4	10	58.82%	7	41.18%
CRIq level 5	9	90.00%	1	10.00%

Table 5. MCI distribution. Abbreviations: MCI: Mild cognitive impairment; iRBD+MCI: iRBD patients with MCI; iRBD-MCI: iRBD patients without MCI; CRIq: Cognitive Reserve Index questionnaire.

3.1.2.4. Association between Neuropsychological Performances and CRIq Levels

Table 6 presents the results of the univariate ANOVA comparing groups defined according to CRIq levels on the mean neuropsychological scores (controlling for disease duration). Results showed significant differences in the scores obtained on the RAVLT immediate recall and the ROCF recall. However, for the RAVLT immediate recall, none of the post hoc pairwise comparisons remained significant after the Bonferroni correction. As concerns ROCF recall, post hoc comparisons showed a significant difference between CRIq level 4 group (mean: 15.41 ± 4.49) and CRIq level 5 group (mean: 21.09 ± 6.09)

with a p-value of 0.024.

Tests	*Pathological cut-offs*	*All patients* $n = 55$	CRIq level 2 $n = 3$	CRIq level 3 $n = 25$	CRIq level 4 $n = 17$	CRIq level 5 $n = 10$	
		Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	*p*
MMSE	< 24.00	28.13 ± 1.39	27.97 ± 2.12	28.49 ± 1.41	27.91 ± 1.57	27.84 ± 0.55	0.638
Token Test	< 26.50	32.01 ± 1.89	33.12 ± 2.50	31.44 ± 2.30	32.51 ± 1.46	31.86 ± 1.09	0.204
Semantic Fluency	< 25.00	45.71 ± 8.81	49.50 ± 11.15	42.73 ± 9.92	45.61 ± 6.38	50.45 ± 7.46	0.107
Phonemic Fluency	< 17.00	32.13 ± 10.39	37.50 ± 16.92	29.09 ± 8.24	33.83 ± 12.68	33.45 ± 6.50	0.405
Oral Naming Test	< 41.99	47.53 ± 0.91	47.85 ± 0.29	47.34 ± 1.14	47.39 ± 0.91	48.00 ± 0.00	[a]
Digit Forward	< 4.26	5.79 ± 1.05	6.16 ± 2.15	5.47 ± 1.09	5.89 ± 0.89	6.17 ± 0.55	0.354
Digit Backward	< 2.65	4.40 ± 1.08	4.56 ± 0.93	4.16 ± 0.95	4.48 ± 1.19	4.70 ± 1.20	0.642
Corsi Span Test	< 3.46	5.21 ± 0.90	5.49 ± 0.75	5.01 ± 1.05	5.43 ± 0.88	5.12 ± 0.64	0.296
RAVLT-I	< 28.53	44.08 ± 7.99	47.85 ± 9.38	40.13 ± 8.72	46.07 ± 6.04	47.34 ± 6.14	0.026*
RAVLT-D	< 4.69	9.62 ± 2.43	10.17 ± 2.35	8.87 ± 2.83	9.76 ± 2.03	10.68 ± 1.95	0.203
RAVLT-R	< 8.00	13.87 ± 2.18	15.75 ± 4.35	13.36 ± 2.63	14.00 ± 0.84	14.00 ± 1.48	0.221
ROCF-C	< 28.88	32.29 ± 3.28	32.31 ± 6.72	32.26 ± 3.05	31.72 ± 3.30	33.25 ± 2.22	0.648
ROCF-R	< 9.47	17.22 ± 5.37	20.37 ± 5.45	16.19 ± 4.73	15.41 ± 4.49	21.09 ± 6.09	0.029*
RPM	< 18.00	29.70 ± 4.20	33.87 ± 2.87	28.75 ± 4.58	29.53 ± 3.92	30.39 ± 3.60	0.066
Attentive Matrices	< 31.00	49.06 ± 5.24	52.00 ± 5.50	49.62 ± 4.24	47.75 ± 6.35	49.02 ± 5.07	0.264

Table 6. Univariate ANOVA between groups identified according to CRIq level on single neuropsychological tests (controlling for disease duration). Abbreviations: CRIq: Cognitive Reserve Index questionnaire; SD: standard deviation; MMSE: mini-mental state Examination; RAVLT-I: Rey auditory verbal learning test-immediate; RAVLT-D: Rey auditory verbal learning test—delayed; RAVLT-R: Rey auditory verbal learning test—recognition; ROCF-C: Rey–Osterrieth complex figure—copy; ROCF-R: Rey–Osterrieth complex figure—recall; RPM: Raven's progressive matrices. [a] The variance in oral naming test is equal to 0 after grouping by CRIq level. * p-value < 0.05.

Table 7 presents the results of the univariate ANOVA comparing groups defined according to CRIq levels on the global z-scores of the five cognitive domains (controlling for disease duration). This analysis showed that the four groups differed significantly in the domains of language (p-value = 0.048) and memory (p-value = 0.032). Nevertheless, for neither the language domain nor the memory domain did any post hoc pairwise comparisons remain significant after the Bonferroni correction.

Cognitive Domain	CRIq Level 2	CRIq Level 3	CRIq Level 4	CRIq Level 5	
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	*p*
Cognitive screening	−0.11 ± 1.51	0.26 ± 0.99	0.15 ± 1.12	−0.20 ± 0.39	0.538
Language	0.40 ± 0.69	−0.22 ± 0.63	0.08 ± 0.42	0.25 ± 0.32	0.048 *
Memory	0.36 ± 0.65	−0.19 ± 0.58	0.06 ± 0.31	0.25 ± 0.38	0.032 *
Executive functions	0.67 ± 0.96	−0.02 ± 0.66	−0.05 ± 0.54	0.05 ± 0.37	0.122
Visual spatial abilities	0.29 ± 2.02	0.28 ± 0.92	0.12 ± 0.99	0.57 ± 0.67	0.458

Table 7. Univariate ANOVA between groups identified according to CRIq level on global

neuropsychological performances (controlling for disease duration). Abbreviations: CRIq: Cognitive Reserve Index questionnaire; SD: standard deviation; * p-value < 0.05.

Lastly, no significant correlation was found. Indeed, correlation analyses between corrected neuropsychological scores and CRIq values (total and subscores) did not show statistically significant results. Likewise, CRIq values and corrected neuropsychological scores did not significantly correlate with disease duration.

3.1.3. Discussion

The present study aimed to evaluate (for the first time) the influence of CR (measured by the CRIq) on the cognitive performance of PSG-confirmed iRBD patients. iRBD represents a unique time window, ideal for testing new neuroprotective methods and better understanding α-synucleinopathy-related neurodegenerative mechanisms. These patients have a variable timing of phenoconversion, ranging from five to 15 years of disease duration. Thus, evaluating the influence of CR on cognitive functioning might be helpful both to explain the inter-subject variability in the onset of cognitive symptoms and to implement strategies to postpone cognitive deterioration based on neuropsychological interventions. We found that iRBD patients with a high-level of CR showed: (i) the lowest percentage of MCI (10 %), and (ii) the best performance in visuo-constructive and verbal memory (the recall of the ROCF test) functions.

The distribution of iRBD+MCI patients across CRIq levels showed a very low percentage of iRBD+MCI subjects within the CRIq level 5 group (high-level CR). This can indicate that an extremely high CR level represents a protective factor against the manifestation of cognitive decline, most likely delaying its onset. Thus, in line with the CR hypothesis (Perneczky et al., 2019; Stern, 2009), our first preliminary results suggest that CR could mitigate the impact of cognitive decline when CRIq scores are at the highest level. This finding is consistent with other studies investigating the CRIq in the context of PD and cognitive impairment, also reporting the same protective role for high CRIq scores (Devos et al., 2023; Guzzetti et al., 2019). In this framework, a recent study by Guzzetti and collaborators (Guzzetti et al., 2019) investigated the role of CR in PD patients by means of the CRIq, supporting not only a protective role of CR against cognitive decline in PD, but also highlighting a prominent beneficial effect of CR at the

later stages of the disease. This could provide evidence that the effect of CR in the early stages of α-synucleinopathies could not be equally strong (as demonstrated in PD) due to the time window of reference. Moreover, it is worth noting that most iRBD patients in the present sample had a high CR level (median: 116.00 ± 18.66); consequently, there were only few subjects in the medium−low CR group (level 2) and none in the low CR group (level 1). This could have led to a ceiling effect, reducing the possibility of observing the effect of CR across all possible levels.

Comparisons of neuropsychological performances between groups classified according to CRIq levels revealed only two statistically significant differences on the RAVLT immediate recall and on the ROCF recall. Both tests belong to the memory domain, which therefore appears to be the most influenced by CRIq scores. This finding was also confirmed by comparisons between the groups on global domains scores, which showed a statistically significant difference in the memory domain, but also in the language domain (which is also implicated in the RAVLT immediate-recall performance). A link between CRIq scores and the memory domain has also emerged in several other studies focusing on other clinical populations (Devos et al., 2023; Guzzetti et al., 2019; Krch et al., 2019; Sandry et al., 2015). This finding might be due to the fact that CRIq gives much weight to activities that promote memory rather than any other cognitive function. Specifically, the CRIq includes the education proxy, but also the working-activity proxy, which is clustered according to the degree of intellectual demand. Indeed, job classification seems to consider the academic preparation required without considering other skills that more manual jobs tend to stimulate. However, it is also important to note that almost all the comparisons did not remain statistically significant after the Bonferroni correction. For this reason, conclusions that can be drawn from these data should be taken with caution.

The present work had several limitations. First, the small sample size severely limited the interpretation of our findings. This also led to a strong imbalance in terms of numerosity for each group identified according to CRIq levels. Thus, our results need to be confirmed by future studies with larger samples equally distributed among the different CRIq levels. Second, the sample was not balanced by gender, which is partly due to the male prevalence of iRBD, but female subjects are still underrepresented. Third, the study's cross-sectional design only provided a snapshot of a dynamic process. Given that

the influence of CR on neuropsychological functioning changes with the progression of the disease (Guzzetti et al., 2019), future longitudinal studies may provide greater insights into the timing of cognitive impairment in iRBD patients with different CR levels. Finally, the CRIq tool itself presents some issues that should be considered. Firstly, job clustering does not take into account the soft skills that some more practical jobs promote, giving almost sole weight to the academic preparation required by different jobs. Secondly, the CRIq tool was created in 2012 (Nucci et al., 2012) and an update might be considered. Indeed, over the last ten years, the use of technology has occupied an increasingly important role in our lives, and therefore, its influence cannot be summarized in one generic item. For these reasons, future validation studies should consider a new, updated version of the CRIq.

To sum up, despite several methodological and practical limitations, this represents the first exploratory study investigating the role of CR in PSG-confirmed iRBD patients by means of a validated questionnaire. Conclusions that can be drawn from the present findings are limited. Still, they highlight the importance of investigating the moderating role of CR on neuropsychological functioning years before a firm diagnosis of neurodegenerative diseases. Future studies should combine CR and BR measures in order to evaluate their interactions across different stages of pathology.

3.2. Study II. Cerebral metabolism, SW and CR in iRBD: towards a functional protective model for the prodromal stage of α-synucleinopathies

Given the premises and the results of the previous study (D'Este et al., 2023), the aim of this second work was to combine CR and BR measures in order to evaluate their interaction and influence on pathological burden and cognitive performance in the prodromal stages of α-synucleinopathies.

As described in detail before (paragraph 3.1), the CR hypothesis states that individuals with a high reserve have greater neural resources, requiring more pathology burden to reach the critical threshold needed to clinically manifest ongoing pathological mechanisms (thus delaying the onset of clinical symptoms) (D'Este et al., 2023; Tucker & Stern, 2011). In this context, the identification of proxies for the quantification of pathological burden in iRBD is of outmost importance. One good candidate is the PDRP,

which represents a marker of local synaptic and neural dysfunction expressed in a pattern-specific manner characteristics of PD (see paragraph 1.2.4.1.) (Ma et al., 2007; Teune et al., 2014). The PDRP is identified using [^{18}F]FDG–PET imaging and it is a reliable biomarker for the quantification of prodromal features of conversion in iRBD, able to differentiate patients from HC (Carli, et al., 2020; Carli et al., 2023). Equally important can be identifying neurophysiological characteristics that may interact with pathological burden and CR. In this context, SW sleep may play a crucial role since it is considered a protective factor from neurodegeneration and cognitive decline (see paragraph 1.2.4.2.) (Cordone et al., 2019; Gorgoni & Galbiati, 2022; Ju et al., 2017; Mander et al., 2015). However, while SW sleep is renowned for its protective effects against degeneration, its specific role in α-synucleinopathy-related neurodegeneration remains poorly understood. Moreover, also the relationship between SW and PDRP is unexplored.

Given these premises, the main objective of the present study was to investigate the relationship between SW and PDRP alongside cognitive performance in iRBD, with an exploratory focus on CR. In more detail, three specific aims were established: i) to confirm the relationship between SW sleep and cognitive impairment; ii) to explore the relationship between SW sleep and PDRP; and iii) to explore the combined impact of CR and SW indexes on pathological burden and cognitive performance.

3.2.1. Methods

3.2.1.1. Participants

Forty-one PSG-confirmed iRBD patients were retrospectively recruited from the clinical database of the Sleep Disorders Center, San Raffaele Hospital, Milan (Italy). The clinical diagnosis of iRBD was made by a sleep medicine expert according to the third edition of the ICSD (American Academy of Sleep Medicine, 2014). All patients underwent a comprehensive neurological and clinical evaluation including video-PSG recording, neuropsychological assessment, CRIq (Nucci et al., 2012), motor and non-motor symptoms questionnaires (i.e., UPDRS, SCOPA, NMSQ) (Chaudhuri et al., 2006; Goetz et al., 2008; Visser et al., 2004). Notably, eight out of 38 iRBD patients did not perform UPDRS and NMSQ questionnaires; meanwhile, four out of 38 iRBD patients did not perform the SCOPA questionnaire.

Patients were free from dementia, parkinsonian signs, psychiatric or other neurological

disorders (American Psychiatric Association, 2013; Postuma et al., 2015).

3.2.1.2. Video-PSG

The video-PSG examination was performed according to the international 10–20 system (Klem, 1999) using a 19-channel EEG. In addition, the system also included EMG of the submentalis, flexor digitorum superficialis and anterior tibialis muscles (i.e., SINBAR montage) (Frauscher, et al., 2012), two EOG channels (i.e., right and left), ECG, and airflow and respiratory-effort channels. Sleep was manually scored by a sleep medicine expert based on the AASM criteria (Berry et al., 2012).

3.2.1.3. SW and SO detection and indexes computation

SW (1-4 Hz) and SO (0.3-1 Hz) were detected using the SW detection algorithm (https://raphaelvallat.com/yasa/build/html/generated/yasa.sw_detect.html?highlight=sw#yasa.sw_detect) of the "Yet Another Spindle Algorithm" (YASA, version 0.6.1) toolbox for Python (version 3.11) (Carrier et al., 2011; Massimini et al., 2004; Vallat & Walker, 2021). Both SW and SO had to present a minimum peak-to-peak amplitude of 75 μV and were identified only on artefacts free N2 and N3 epochs on frontal EEG derivations (F3, F4). Once waves detection was completed, SW density (SWd) and SO density (SOd) indexes were computed as follow:

$$SWd = \frac{total\ number\ of\ SW\ detected\ in\ N2\ or\ N3\ sleep}{min\ of\ N2\ or\ N3\ sleep}$$

$$SOd = \frac{total\ number\ of\ SO\ detected\ in\ N2\ or\ N3\ sleep}{min\ of\ N2\ or\ N3\ sleep}$$

3.2.1.4. [^{18}F]FDG–PET image acquisition and pre-processing

Based on the European Association Of Nuclear Medicine (EANM) guidelines (Varrone et al., 2009), all iRBD patients and HC underwent [^{18}F]FDG-PET imaging with Discovery STE CT-PET scanner (3.27 mm thickness; 5.55 mm in-plane FWHM; GE Healthcare medical systems). A 256×256 matrix with a pixel size of 1.95 mm was used for image acquisition with 47-views, in 10–15 minutes for a total count of 200×10. An ordered subset-expectation maximization algorithm was used for image reconstruction.

Data pre-processing was performed using the MATLAB (The MathWorks, Inc., Natick, MA) based software "Statistical parametric mapping" (SPM, version 12). Each [^{18}F]FDG-PET scan has been visually inspected, and the origin was manually set. Subsequently, all images were spatially normalized to a [^{18}F]FDG-PET template in Montreal neurological institute (MNI) brain space (Della Rosa et al., 2014). Image smoothing was performed using an isotropic 3D Gaussian kernel (FWHM: 8-8-8 mm). Intensity normalization was performed using the scaled subprofile model (SSM), which is part of the multivariate scaled subprofile model/principal component analysis (SSM/PCA) (Eidelberg, 2009).

3.2.1.5. iRBD subject expression of PDRP

The multivariate SSM/PCA (Eidelberg, 2009) was used to compute PDRP expression scores for each iRBD subject. SSM/PCA was applied using MATLAB-based software developed in-house at the University Medical Center Groningen (UMCG) (Teune et al., 2014), following the methods outlined by Spetsieris and Eidelberg (Spetsieris & Eidelberg, 2011). PDRP was previously identified in [^{18}F]FDG-PET brain images of 17 HC (M/F 12/5, mean age 61.50 ± 7.50 years) and 19 PD patients (M/F 13/6, mean age 63.90 ± 7.80) in the off-L-DOPA state, from the UMCG (Teune et al., 2014). Using the validated PDRP, we calculated subject scores for each iRBD patient and a group of HC. In the SSM/PCA process, the images were initially log-transformed. We then subtracted both the individual subject means and the overall group mean (from the pattern identification cohort) to create a residual profile for each subject. Finally, the subject score was determined by projecting this residual profile on the pattern. The iRBD subject scores were then z-scored on HC group, calculated as follows:

$$z - score = \frac{iRBD\ subject\ score - mean\ (HC\ subject\ scores)}{SD\ (HC\ subject\ score)}$$

3.2.1.6. Neuropsychological Evaluation

At the time of the diagnosis, an expert neuropsychologist administered a comprehensive neuropsychological battery to all iRBD patients (duration: ~1 h). Specifically, the cognitive evaluation included different tests: MMSE (Measso et al.,

1993) (cognitive screening domain); token test (De Renzi & Faglioni, 1978), semantic and phonemic verbal fluency tests (Novelli et al., 1986), CAGI oral denomination test (Catricalà et al., 2013) (language domain); digit-span test (forward (Orsini et al., 1987) and backward (Kessels et al., 2008) versions), Corsi block-tapping test (Spinnler & Tognoni, 1987), RAVLT test (immediate recall, delayed recall and recognition parts) (Carlesimo et al., 1996), recall of the ROCF test (Caffarra et al., 2002) (memory domain); RPM test (Spinnler & Tognoni, 1987), attentive matrices test (Spinnler & Tognoni, 1987), trail making test (TMT – A, B, B-A parts) (Giovagnoli et al., 1996) (executive function domain); and copy of the ROCF test (Caffarra et al., 2002) (visuospatial abilities domain). Notably, only 20 out of 41 patients performed the TMT test (A, B, B-A parts) (Giovagnoli et al., 1996). After the cognitive evaluation, the scores obtained in each neuropsychological test were adjusted for age, gender and education based on national normative values (corrected scores). Based on these, global cognitive domain scores were computed by z-transforming the corrected scores of each neuropsychological test and averaging them separately based on the corresponding cognitive domain (i.e., cognitive screening, memory, language, executive function and visuospatial abilities).

The presence of MCI was evaluated in all patients and a clinical diagnosis was made according to the criteria proposed by Petersen and colleagues in 2018 (Petersen et al., 2018). In more detail, MCI was classified according to both its nature and the number of impaired cognitive domains (Petersen, 2004; Petersen et al., 2014). Concerning MCI nature, patients were classified as aMCI if they showed deficits on the memory cognitive domain; on the opposite, if they showed impaired performance in other cognitive domains (apart from memory) they were classified as naMCI (Petersen, 2004; Petersen et al., 2014). Regarding the number of impaired cognitive domains, patients were classified as MCIsd if they showed deficits in a single cognitive domain; on the opposite, when multiple domains were affected, patients were classified as MCImd (Petersen, 2004; Petersen et al., 2014).

3.2.1.7. CRIq

The CRIq was administered to all the patients included in the present study. The CRIq is a validated questionnaire able to quantify CR throughout the lifespan by means of three different proxies: education, working and leisure time activities (Nucci et al., 2012). Each

activity is evaluated based on the number of years it has been carried out and its regularity. Based on these data, one composite total score (and three subscores) is computed and classified into five different levels: 1) low CR (≤ 70); 2) medium–low CR (70–84); 3) medium CR (85–114); 4) medium–high CR (115–130); 5) high CR (≥ 130) (Nucci et al., 2012).

3.2.1.8. Statistical Analyses

The data analysis was performed using JASP (version 0.19.1). Descriptive statistics were obtained for variables including age, gender, educational level, disease duration, sleep indexes, cognitive assessments, MCI presence and characterization, CRIq, as well as motor and non-motor symptom questionnaires.

Comparisons on neuropsychological, PDRP and sleep data were performed between iRBD-MCI and iRBD+MCI patients. The Shapiro–Wilk test was used to verify data normality. Based on variables distribution, the Student's t-test or the Mann-Whitney U test were applied. Moreover, correlations between SW sleep, PDRP and neuropsychological data were performed. Based on variables distribution, parametric (Pearson's r) or non-parametric correlations (Spearman's rho) were used.

Finally, a mediation analysis was performed. In more detail, the mediation analysis was conducted using the Delta method for standard errors and normal theory confidence intervals, applying a Maximum Likelihood (ML) estimator. The analysis aimed to assess both direct and indirect effects of PDRP expression (i.e., predictor variable) on cognitive screening performance (i.e., outcome variable), with potential mediation through two intermediate variables: CRIq score and SWd in N3 sleep.

For all the analyses, p-values less than 0.05 were considered statistically significant.

3.2.2. Results

3.2.2.1. Descriptive Statistics

The descriptive statistics of the whole sample are illustrated in Table 8. A total of 41 iRBD patients (32 males, 78.05 %) were included in the study. iRBD patients had a mean age of 66.15 ± 8.14 years, a mean education of 12.00 ± 4.03 years and a mean disease duration of 7.24 ± 3.61 years – this latter was computed as the time between subjective symptoms onset and the time of diagnosis. Sleep was characterized by prolonged REM

sleep latency (mean: 141.12 ± 54.63 min), high variability in N1 (mean: 11.65 ± 4.09 %) and N3 (mean: 20.68 ± 8.94 %) sleep, and a moderate sleep efficiency (mean: 76.06 ± 11.43 %). Sixteen out of 41 patients had MCI (39.02 %), specifically most patients were affected by naMCI (75.00 %) and MCIsd (68.75 %). Concerning CR characteristics, patients showed a medium CRIq total score of 111.49 ± 17.41, indeed most patients were classified as having a medium-high (41.46 %) followed by a medium (36.59 %) level of CR. Finally, regarding motor and non-motor symptoms, patients showed a UPDRS total score of 7.42 ± 5.83, a SCOPA-AUT total score of 8.16 ± 6.58 and a NMSQ total score of 25.03 ± 25.81, with only 14 out of 41 patients reporting hyposmia.

	n	**%**
Males	32	78.05%
Hyposmia (yes)	14	34.15%
CRI-Level		
Level 1	0	0.00%
Level 2	4	9.76%
Level 3	15	36.59%
Level 4	17	41.46%
Level 5	5	12.20%
MCI (yes)	16	39.02%
MCI (no)	25	60.98%
aMCI	4	25.00%
naMCI	12	75.00%
MCIsd	11	68.75%
MCImd	5	31.25%
	Mean ± SD	**Min-Max**
Age (years)	66.15 ± 8.14	50.00-82.00
Years of education (years)	12.00 ± 4.03	5.00-18.00
Disease duration	7.24 ± 3.61	3.00-21.00
N1 Latency (min)	32.33 ± 24.53	0.00-123.00
N2 Latency (min)	43.43 ± 28.96	13.50-125.50
N3 Latency (min)	77.24 ± 51.88	22.50-300.00
REM Latency (min)	141.12 ± 54.63	59.00-277.50
N1 (%)	11.65 ± 4.09	3.78-23.24
N2 (%)	47.56 ± 8.50	29.09-64.19
N3 (%)	20.68 ± 8.94	6.86-48.48
REM (%)	20.11 ± 7.01	6.24-36.79
NREM (%)	79.89 ± 7.01	63.21-93.77
SE (%)	76.06 ± 11.43	41.86-91.05
UPDRS total score	7.42 ± 5.83	0.00-22.00
SCOPA total score	8.16 ± 6.58	0.00-26.00
NMSQ total score	25.03 ± 25.81	0.00-117.00
CRI-Education	106.59 ± 13.20	84.00-127.00
CRI-Working Activity	110.56 ± 17.20	72.00-142.00
CRI-Leisure Time	108.85 ± 20.32	72.00-148.00
CRI-Total	111.49 ± 17.41	77.00-148.00

Table 8. Descriptives statistics of the whole sample. Abbreviations: aMCI, amnestic mild cognitive impairment; CRI, Cognitive Reserve Index; max, maximum; MCI, mild cognitive

impairment; MCImd, mild cognitive impairment multi-domain; MCIsd, mild cognitive impairment single-domain; naMCI, non-amnestic mild cognitive impairment; min, minimum; NMSQ, Non-Motor Symptoms Questionnaire; NREM, non-rapid eye movement; N1, stage-one sleep; N2, stage-two sleep; N3, stage-three sleep; REM, rapid eye movement; SCOPA, Scales for Outcomes in Parkinson's Disease; SD, standard deviation; min, minutes; SE, sleep efficiency; UPDRS, Unified Parkinson's Disease Rating Scale.

3.2.2.2. Comparisons between iRBD patients with and without MCI

iRBD+MCI patients performed significantly worse in all global cognitive domains compared to iRBD-MCI patients i.e., cognitive screening ($p = 0.039$), language ($p = 0.006$), memory ($p = 0.049$), executive function ($p = 0.028$) and visuospatial abilities ($p < 0.001$). In more detail, iRBD+MCI had significantly lower scores in the MMSE ($p = 0.039$), semantic ($p = 0.022$) and phonemic ($p = 0.029$) verbal fluency, digit span forward ($p = 0.027$) and backward ($p = 0.015$), ROCF copy ($p < 0.001$) and recall ($p = 0.007$) tests compared to iRBD-MCI patients. Table 9 illustrates the neuropsychological characteristics of the whole sample and the comparisons between iRBD-MCI and iRBD+MCI on neuropsychological tests.

Neuropsychological tests	Pathological Cut-Offs	All Patients n = 41 (mean ± SD)	iRBD-MCI n = 25 (mean ± SD)	iRBD+MCI n = 16 (mean ± SD)	p-value
MMSE	< 24.00	28.01 ± 1.40	28.37 ± 1.08	27.45 ± 1.69	p = 0.039
Token test	< 26.50	31.93 ± 2.06	32.24 ± 1.59	31.42 ± 2.66	NS
Semantic fluency	< 25.00	44.88 ± 8.93	47.40 ± 9.26	40.94 ± 6.95	p = 0.022
Phonemic fluency	< 17.00	32.61 ± 11.23	35.64 ± 12.08	27.88 ± 7.97	p = 0.029
Oral naming test	< 41.99	47.55 ± 0.81	47.66 ± 0.77	47.35 ± 0.87	NS
Digit forward	< 4.26	5.83 ± 1.12	6.22 ± 1.08	5.22 ± 0.93	p = 0.027
Digit backward	< 2.65	4.34 ± 1.78	4.69 ± 1.22	3.79 ± 0.88	p = 0.015
Corsi span test	< 3.46	5.17 ± 0.91	5.22 ± 0.95	5.10 ± 0.88	NS
RAVLT-I	< 28.53	44.09 ± 7.84	46.14 ± 6.62	40.87 ± 8.69	NS
RAVLT-D	< 4.69	9.30 ± 2.34	9.54 ± 2.09	8.94 ± 2.73	NS
RAVLT-R	< 8.00	13.76 ± 2.52	13.68 ± 1.55	13.88 ± 3.61	NS
ROCF-C	< 28.88	31.96 ± 3.54	33.71 ± 1.80	29.22 ± 3.88	p < 0.001
ROCF-R	< 9.47	17.40 ± 5.10	19.07 ± 4.95	14.78 ± 4.26	p = 0.07
RPM	< 18.00	29.73 ± 4.03	30.66 ± 3.63	28.28 ± 4.31	NS
Attentive matrices	< 31.00	49.31 ± 4.85	50.36 ± 5.04	47.67 ± 4.18	NS
TMT A	> 93	24.60 ± 13.29	23.67 ± 12.40	26.00 ± 15.30	NS
TMT B	> 282	70.20 ± 27.88	69.42 ± 25.66	71.38 ± 32.75	NS
TMT B-A	> 186	45.90 ± 29.36	46.00 ± 26.70	45.75 ± 32.59	NS

Table 9. Neuropsychological characteristics of the whole sample and comparisons between iRBD-MCI and iRBD+MCI on neuropsychological tests. Abbreviations: iRBD, isolated Rapid Eye-Movement Sleep Behavior Disorder; iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; MMSE, Mini-Mental State Examination; NS, not significant; RAVLT-I; Rey auditory verbal learning test – immediate recall;

RAVLT-D; Rey auditory verbal learning test – delayed recall; RAVLT-R; Rey auditory verbal learning test – recognition; ROCF-C, copy of the Rey–Osterrieth complex figure; ROCF-R, recall of the Rey–Osterrieth complex figure; RPM, Raven Progressive Matrices test; SD, standard deviation; TMT A, Trail Making Test part A; TMT B, Trail Making Test part B; TMT B-A, Trail Making Test part B-A;.

Moreover, iRBD-MCI and iRBD+MCI patients were also compared on SW sleep indexes. Specifically, iRBD+MCI patients showed a significant reduction in both SWd in N2 ($p = 0.006$) and N3 sleep ($p = 0.006$) and in SOd in N2 ($p=0.036$) and N3 sleep ($p=0.028$) compared to iRBD-MCI (see Figure 9).

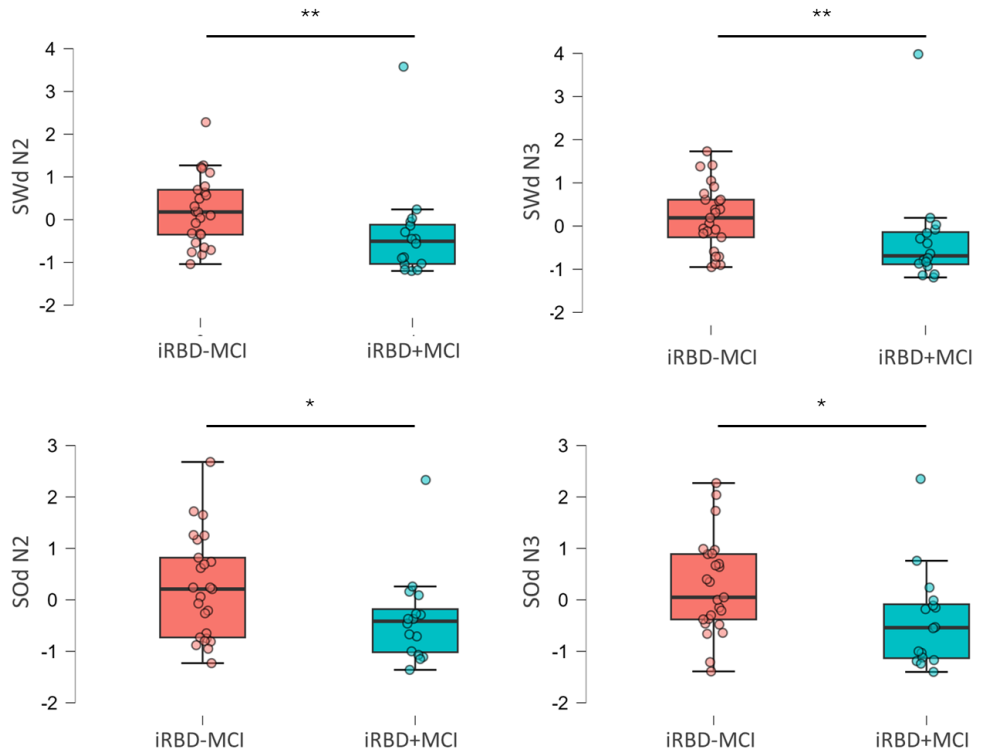


Figure 9. Comparisons between iRBD-MCI and iRBD+MCI patients on SW sleep indexes. * indicate the level of significance. Abbreviations: iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; N2, stage-two sleep; N3, stage-three sleep; SOd, slow oscillations density; SW, slow waves density.

Notably, iRBD+MCI showed a non-significant reduction in CRIq compared to iRBD-MCI. Additionally, there were no significant differences between the two groups in PDRP expression.

3.2.2.3. Correlation Analysis

Correlation analyses were performed between SW sleep indexes and neuropsychological data (see Figure 10). Regarding global cognitive domains, the results showed that SWd in N3 sleep was significantly positively associated with cognitive screening (r = 0.335, p = 0.032) and visuospatial abilities (r = 0.405, p = 0.009) domains; of note, this latter was also significantly positively associated with SWd in N2 sleep (r = 0.321, p = 0.041) and with SOd in N3 sleep (r = 0.345, p = 0.027). Concerning neuropsychological tests, SWd in N3 sleep was significantly positively associated with the digit span forward (r = 0.317, p = 0.044) and backward (r = 0.358, p = 0.021) tests and with the RPM test (r = 0.331, p = 0.035), which in turn was also significantly positively correlated with SWd in N2 sleep (r = 0.346, p = 0.027). This means that higher SWd or SOd indexes (in N2 or N3 sleep) were associated with better performances in all the abovementioned neuropsychological tests and cognitive domains.

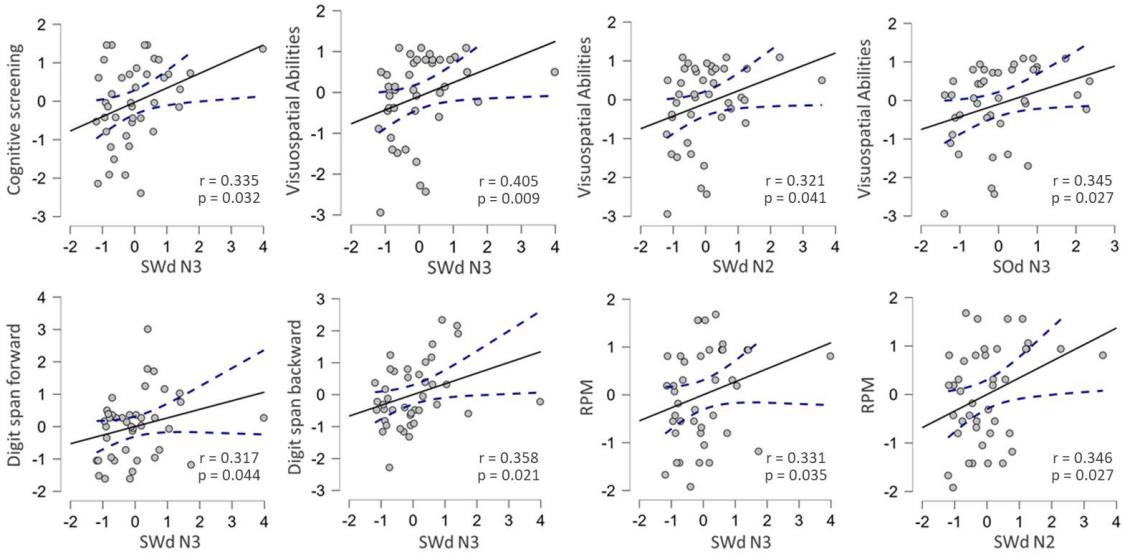


Figure 10. Correlation analysis between SW sleep indexes and neuropsychological data. Abbreviations: N2, stage-two sleep; N3, stage-three sleep; RPM, Raven Progressive Matrices test; SOd, slow oscillations density; SWd, slow waves density.

The results of the correlation analysis performed between PDRP expression and SW sleep indexes showed a significant negative association between PDRP expression and SWd in N3 sleep (r = –0.318, p = 0.043) (see Figure 11). That is, the higher the expression of the PDRP, the lower the SWd in N3 sleep.

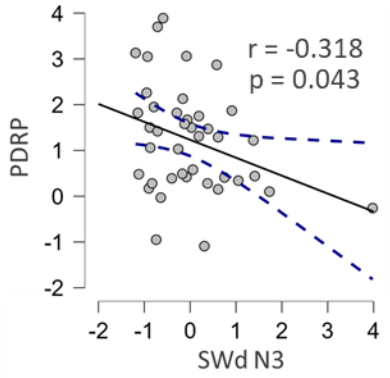


Figure 11. Correlation analysis between PDRP and SWd in N3 sleep. Abbreviations: N3, stage-three sleep; PDRP, Parkinson's Disease Related Pattern; TMT.

Moreover, the correlation analysis performed between PDRP expression and neuropsychological data showed only one positive significative association between PDRP expression and TMT A score (r = 0.491, p = 0.028) (see Figure 12). This means that a higher expression of the PDRP was associated with worse performances in the TMT A – indeed, the TMT A score is the only neuropsychological score expressed in seconds, so a higher score reflects worse performances.

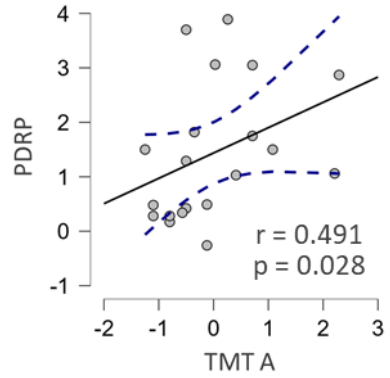


Figure 12. Correlation analysis between PDRP and TMT A. Abbreviations: PDRP, Parkinson's Disease Related Pattern; TMT, Trail Making Test part A.

Of note, the correlation analysis performed between SW sleep indexes, PDRP expression and CRIq scores showed no significant result.

3.2.2.4. Mediation Analysis

Finally, a mediation analysis using the Delta method was performed. This analysis did

not reveal any significant direct or indirect effects. The direct effect between the predictor (i.e., PDRP expression) and the outcome (i.e., cognitive screening score) was not significant, showing an estimate of -0.082 (p = 0.547). Moreover, both indirect effects were also not significant. Indeed, the path mediated by the CR score had an estimate of 0.009 (p = 0.725); meanwhile the path via SWd in N3 sleep had an estimate of -0.098 (p = 0.118). Additionally, there was no significance in residual covariances, further supporting the idea that the tested mediators did not play a substantial role in the relationship between the predictor and the outcome variables. Notably, also the total effect (estimate = -0.171, p = 0.214) and the total indirect effects (estimate = -0.089, p = 0.184) did not reach significance. However, when examining the path coefficients, it was observed a significant effect of SWd in N3 sleep not only on the outcome (estimate = 0.354, p = 0.029), but also on the predictor (estimate = -0.278, p = 0.025). Figure 13 illustrates the path plot of the mediation analysis.

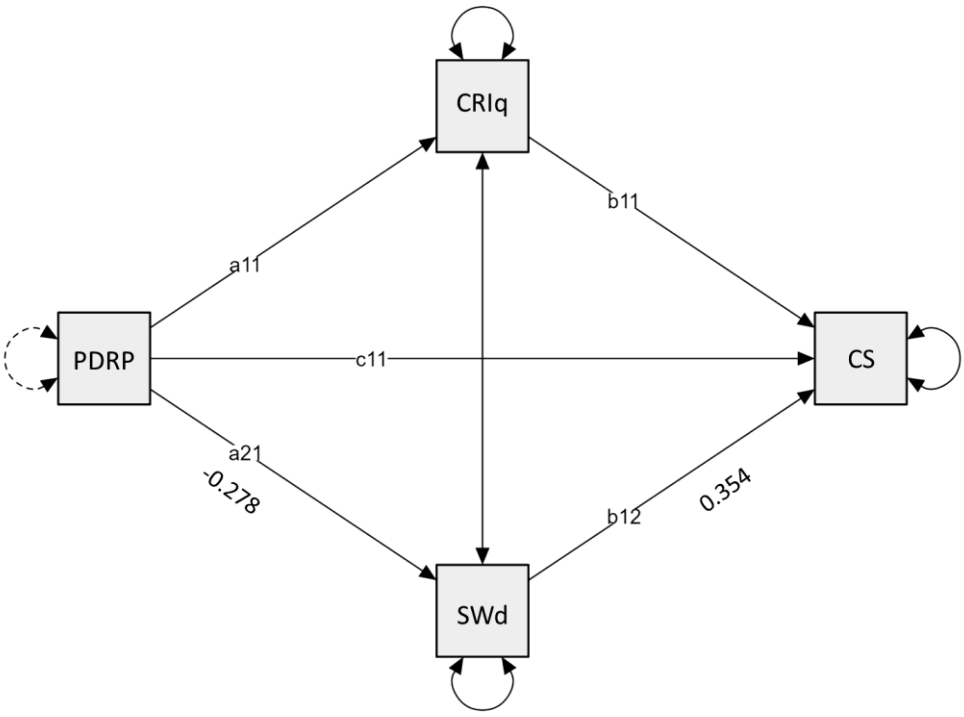


Figure 13. Mediation analysis using Delta method. The predictor variable is the PDRP expression. The outcome variable is the cognitive screening score. The first mediator variable is the CRIq; meanwhile, the second mediator variable is the SWd in N3 sleep. a11 defines the direct relation between the predictor and the first mediator. b11 defines the direct relation between the first mediator and the outcome variable. a21 defines the direct relation between the predictor and the second mediator. b12 defines the direct relation between the second mediator and the outcome variable. c defines the direct effect of the predictor variable on the outcome variable (statistically

controlling for the effect of the two mediators). Abbreviations: CRIq, Cognitive Reserve Index questionnaire; CS, cognitive screening; PDRP, Parkinson's Disease Related Pattern; SOd, slow oscillations density; SWd, slow waves density.

3.2.3. Discussion

Starting from the results of our previous work (D'Este et al., 2023), the present study aimed to combine CR and BR measures in order to evaluate their interaction and influence on pathological burden and cognitive performance in a cohort of PSG-confirmed iRBD patients. The results not only replicated the relationship between SW and cognitive impairment, but they also demonstrated the presence of an inverse association between PDRP expression and SWd in N3 sleep. However, no significant relation was found between PDRP expression, SW sleep indexes, cognitive functioning, and CR, providing no support for the potential role of CR in this context.

The results demonstrated that iRBD+MCI patients showed a significant reduction in SWd and in SOd (in both N2 and N3 sleep) compared to iRBD-MCI. In line with AD literature (Cordone et al., 2019; Gorgoni & Galbiati, 2022; Mander et al., 2015), previous studies on iRBD reported not only a reduction in SW and SO (Sunwoo et al., 2021; Valomon et al., 2021), but also an important reduction in KC (forerunner of SW) which was specifically associated with the presence of MCI (Galbiati et al., 2021) (see paragraph 1.2.4.2.). This evidence is further supported by correlation analyses showing a significant positive association between SWd (both in N2 and N3 sleep) and SOd (in N3 sleep) and the cognitive performance in cognitive screening and visuospatial abilities domains. This finding is in line with previous results of our laboratory (Galbiati et al., 2021) reporting that KCd was significantly positively correlated with global cognitive functioning, specifically visuospatial abilities scores. These findings also suggest that SW a SO may be a protective factor from both neurodegeneration and cognitive decline, as reported in previous studies (Ju et al., 2017; Mander et al., 2015). The main hypothesis underlying the association between cognitive impairment and the reduction in SW indexes takes root in the pathophysiology of iRBD (see paragraphs 1.1.1. and 1.2.2.). Indeed, iRBD fits in the prodromal stages of the α-synucleinopathies spectrum, reflecting a period during which early indicators and symptoms of neurodegeneration appear, yet they are not pronounced enough to diagnose a full-blown disease, mainly PD and PDD/DLB (Berg et al., 2015). However, pathological mechanisms of these conditions are already in effect in

this prodromal phase, as exemplified by the early spreading of α-syn aggregates through the brain in iRBD (Iranzo et al., 2013; Miglis et al., 2021). Therefore, it can be hypothesized that α-syn pathology spreading in deep brain structures may not only lead to the emergence of RSWA and iRBD (Braak stage II) (Del Tredici & Braak, 2016; Poewe et al., 2017; Silva et al., 2023), but its exacerbation and progression may also contribute to alterations in brain rhythms resulting in SW sleep disruption and consequent appearance of cognitive deficits (Galbiati et al., 2021; Silva et al., 2023). This hypothesis also supports the finding that a higher PDRP expression was associated with lower SWd in N3 sleep in the present study. Indeed, an increased PDRP expression reflects a higher local synaptic and neural dysfunction, which is probably mainly driven by the severity of α-syn pathology and can serve as a measure of pathological burden (Meles et al., 2017). Notably, PDRP expression also showed a significant positive association with TMT A performances, meaning that a higher pathological burden was associated with worse performances in the TMT A. This evidence further highlights the role of both pathological burden and sleep disruption in driving cognitive decline.

Given these premises, a mediation model was built in order to test whether CR and SWd were able to reduce the impact of pathological burden on cognitive performances. The results of the mediation analysis did not indicate significant mediation effects of CR and SWd on the relationship between PDRP and cognitive functioning. However, it was observed a significant path effect of SWd not only on the pathological burden, but also on cognitive performances, thus highlighting the importance of SWd in understanding the relationship between sleep architecture, neurodegeneration and clinical symptoms manifestation in iRBD.

Of note, several limitations should be acknowledged. First, the present study had a cross-sectional nature, thus limiting the interpretability of the results. Second, PDRP expression may not be the best proxy for measuring pathological burden. Indeed, PDRP not only can vary significantly among individuals, but it also may not fully capture the complexity of α-syn pathology, lacking specificity within the context of iRBD. Third, even though CRIq is one of the most comprehensive validated tools for measuring CR, it also presents several issues (see D'Este et al., 2023 for an overview). Thus, the CRIq may not be a reliable outcome for testing the effect CR in this context. For all these reasons, future longitudinal studies should test CR within a multimodal approach combining

different measures for the evaluation of the pathological burden and ideally considering an updated version of the CRIq.

In conclusion, the present study not only confirmed previous evidence linking SW disruption to cognitive impairment, but also suggested a novel interaction between pathological burden and SW sleep dynamics in iRBD. The observed inverse relationship between PDRP expression and SWd underscores the potential role of α-synuclein pathology in disrupting SW sleep, which is also closely tied to cognitive deficits manifestation. Future studies should further investigate the mechanisms by which neurodegenerative processes contribute to SW disruption and to its clinical manifestations, as this could open new therapeutic trajectories for early intervention within the α-synucleinopathies spectrum.

4. COGNITIVE & AUTONOMIC FUNCTIONING: THE PLR IN THE EARLY STAGES OF PROGRESSION TO ALPHA-SYNUCLEINOPATHIES

4.1. Introduction

As previously stated, nowadays iRBD is recognized as a prodromal stage of α-synucleinopathies, providing a unique window for the identification of early predictive biomarkers of progression to a full-blown neurodegenerative disorder (Galbiati et al., 2019). One area of growing interest in recent literature is the ANS and its role in the underlying neurodegenerative processes. Indeed, autonomic dysfunction has been reported not only in patients with PD and PDD/DLB (Bae et al., 2009; Hu et al., 2022), but it is also a prominent feature of iRBD (Chiaro et al., 2018; Ferini-Strambi, Oertel, et al., 2014; Inoue & Sasai-Sakuma, 2019). Regarding the pupillomotor domain, the PLR – defined as the change in pupil size in response to light (Larson & Behrends, 2015) – has emerged as a promising marker of cholinergic dysfunction in these conditions (Fotiou et al., 2009; Stergiou et al., 2009; Sunwoo et al., 2022). Indeed, the PLR is a well-defined physiological response regulated by two key autonomic processes: PANS activation, which drives pupil constriction phase via the iris sphincter muscle, and SANS activation, which drives pupil re-dilation phase via the iris dilator muscle (Wang et al., 2016). Pathological changes within these autonomic systems, particularly in parasympathetic cholinergic pathways, could result in PLR alteration reflecting early cholinergic degeneration. Indeed, severe cholinergic denervation of cortical areas due to degeneration of the nucleus basalis of Meynert has been reported in both PD and PDD/DLB patients, being the main cause of cognitive impairment (Müller & Bohnen, 2013; Okkels et al., 2024; van der Zee et al., 2021). Notably, the progression to dementia in these disorders seems to be mainly driven by a posterior cholinergic dysfunction, resulting in a more severe involvement of the visuospatial abilities domain (Kehagia et al., 2012; Klein et al., 2010; Müller & Bohnen, 2013; Shimada et al., 2009). In line with this evidence, previous pupillometric studies on PD patients demonstrated altered PLR parameters, pointing to an underlying cholinergic deficit (Fotiou et al., 2009; Stergiou et al., 2009). These alterations have been shown to correlate with cognitive decline, further strengthening the hypothesis that PLR could serve as a useful biomarker in neurodegenerative diseases

(Fotiou et al., 2009; Stergiou et al., 2009).

Recent findings suggest that cholinergic dysfunction may also be present in iRBD patients (Carli, et al., 2020; Gersel Stokholm et al., 2020); however, despite growing evidence, the relationship between early cholinergic deficits and cognitive impairment in this population remains underexplored. Moreover, a limited number of studies investigated pupillary functioning in iRBD (La Morgia et al., 2022; Perkins et al., 2021; Sunwoo et al., 2022), among which only one used a portable pupillometer for the evaluation of the PLR (Sunwoo et al., 2022). This last study reported that iRBD patients exhibited an altered PLR comparable to that seen in PD, supporting the presence of an early cholinergic deficiency in the prodromal stages of α-synucleinopathies (Sunwoo et al., 2022). Despite these findings, the lack of data calls for further research assessing the role of PLR within the field of iRBD.

Given the potential of pupillometry to detect early autonomic dysfunction, the present study aimed: i) to investigate the presence of peripheral cholinergic dysfunction in iRBD patients using a portable pupillometer for the evaluation of the PLR; ii) to test whether this autonomic dysfunction is associated with cognitive impairment in iRBD patients, particularly with performances in the visuospatial abilities domain by parceling it using the Visual Object and Space Perception (VOSP) battery.

4.2. Methods

4.2.1. Participants

The present study included 38 iRBD patients and 18 HC, recruited at the Sleep Disorder Centre of the San Raffaele Hospital (Milan, Italy). The diagnosis of iRBD was confirmed through PSG by a sleep-medicine expert based on the third edition of the ICSD (American Academy of Sleep Medicine, 2014). All iRBD patients underwent a comprehensive clinical and neurological evaluation including the administration of several motor and non-motor questionnaires namely the UPDRS (Goetz et al., 2008), the SCOPA-AUT (Visser et al., 2004) and the NMSQ (Chaudhuri et al., 2006). Notably, three out of 38 iRBD patients did not perform the UPDRS.

Both iRBD patients and HC underwent a comprehensive neuropsychological assessment and pupillometry. Based on the cognitive evaluation, the possible presence of MCI was assessed in all subjects based on Petersen's criteria (Petersen et al., 2014).

Notably, one out of 38 iRBD patients did not perform the TMT; moreover, two out of 18 HC were cognitively evaluated using only the MMSE.

Patients with dementia, parkinsonian signs, psychiatric or other neurological disorders were excluded (American Psychiatric Association, 2013; Postuma et al., 2015). Additional exclusion criteria for pupillometry were applied based on previous literature (Fotiou et al., 2009; Stergiou et al., 2009). Specifically, subjects (both HC and iRBD) were not to take any anticholinergic, cholinomimetic, antidepressants, cardiac glycosides, sympathomimetics, beta-blockers, or other medications affecting pupillary reflexes and heart rate variability (Fotiou et al., 2009; Stergiou et al., 2009). Exclusion criteria for pupillometry also included neurological and ophthalmological disorders affecting pupillary reflexes and prior ocular surgeries (Fotiou et al., 2009; Stergiou et al., 2009).

4.2.2. Neuropsychological evaluation

The cognitive evaluation administered to both iRBD patients and HC comprised a neuropsychological battery lasting 1 h and 30 min, which assessed five different cognitive domains: cognitive screening, language, memory, executive function and visuospatial abilities. Notably, the cognitive evaluation of iRBD patients was made at the time of the diagnosis. Specifically, several standardized tests were included in the battery, namely the MMSE (Measso et al., 1993) – cognitive screening domain; the token test (De Renzi & Faglioni, 1978), semantic and phonemic verbal fluency tests (Novelli et al., 1986) and the CAGI oral denomination test (Catricalà et al., 2013) – language domain; the digit-span forward (Orsini et al., 1987) and backward (Kessels et al., 2008) versions, the Corsi block-tapping test (Spinnler & Tognoni, 1987), the immediate recall, delayed recall and recognition parts of the RAVLT (Carlesimo et al., 1996) and the recall of the ROCF (Caffarra et al., 2002) – memory domain; the TMT – part A, B, B-A (Giovagnoli et al., 1996), the RPM (Spinnler & Tognoni, 1987) and the attentive matrices test (Spinnler & Tognoni, 1987) – executive function domain; the copy of the ROCF (Caffarra et al., 2002) and the VOSP battery (Warrington & James, 1991) – visuospatial abilities domain. The VOSP battery included, besides one initial screening subtest, eight different subtests for the evaluation of both visuoperceptive and visuospatial skills (Warrington & James, 1991). On one hand, object perception was evaluated through the incomplete letters, the silhouettes, the object decision and the progressive silhouettes subtests (Warrington &

James, 1991). On the other hand, space perception was evaluated by means of the dot count, the position discrimination, the number location and the cube analysis subtests (Warrington & James, 1991).

Based on Italian norms, the raw scores of each neuropsychological test were adjusted for age, gender and/or years of education resulting in "corrected scores". Moreover, global cognitive-domain scores were computed by z-transforming the corrected scores of each neuropsychological test and averaging them separately based on the corresponding cognitive domain (i.e., cognitive screening, memory, language, executive function and visuospatial abilities). With regard to the visuospatial abilities cognitive domain, global scores of object and space perception were also computed by z-transforming the scores of each VOSP subtests and averaging them separately based on visuoperceptive and visuospatial skills.

4.2.3. Pupillometric evaluation and data processing

The pupillometric evaluation was performed using the portable pupillometry "NeuroLight, version 1.16 – IT" (IDMED, France). This tool comprised a measuring system using a camera and an embedded processor. The measuring range for the pupil diameter was one to ten mm, with a precision of 0.1 mm (min 3%), a resolution of 0.01 mm and temporal resolution of 62 frames per second. Light stimulus duration was one s with an intensity of 320 lux (precision ± 10 %). In line with previous literature, the experimental condition started with two min of complete darkness, after which five rectangular flashes of light were administered at 30 s intervals in each eye alternatively, resulting in a total of ten acquisitions (Fotiou et al., 2009; Stergiou et al., 2009). Notably, in the case of compromised data captures, a few supplementary acquisitions were collected.

Data were processed using the software "NLViewer" (version 1.3.2.20) and the following PLR parameters were extracted: i) base (mm), defined as the baseline pupil diameter before the presentation of the light stimulus; ii) variation (mm), defined as the change in pupil diameter after the presentation of the light stimulus; iii) latency (ms), defined as the time of onset of the pupil reaction to the light stimulus; iv) VCmax (mm/s), defined as the maximum constriction velocity of the pupil (see paragraph 1.2.4.3.). After data extraction, artefacts removal was performed on raw data. In more detail, each

subject's curve was visually evaluated by the same rater (G.D.) in order to detect the possible presence of artefacts affecting the PLR dynamic. Artefact-contaminated acquisitions were then replaced by mediating the previous and following records, resulting in ten fully cleaned acquisitions for each subject. Based on artefacts-free data, PLR curves were computed and averaged for each group separately using RStudio (version 2023.06.0, build 421). Moreover, outliers' detection was also performed to remove other possible confounding that could have affected PLR parameters. The applied criterion for outliers' removal was defined as two standard deviations above/below the mean, which was applied to each subject separately in order not to flatten inter-subject variability.

4.2.4. Statistical Analyses

The data analysis was performed using JASP (version 0.19.1). Descriptive statistics were run for both groups (HC and iRBD) and data regarding age, gender, years of education, cognitive and pupillometric evaluations were collected. Moreover, iRBD patients were also characterized in terms of disease duration, MCI, motor and non-motor symptoms (i.e., UPDRS, SCOPA-AUT, NMSQ) presence.

First, in order to investigate possible changes in PLR dynamics, comparisons between HC and iRBD groups for both neuropsychological and pupillometric variables were performed. The Shapiro–Wilk test was used to verify data normality. Based on variables distribution, the Student's t-test and the Mann-Whitney U test were applied. Since age significantly differed between the two groups, it was entered as a covariate in the group comparisons. Second, in order to verify whether changes in PLR dynamics could have been mediated by the presence of cognitive deficits, comparisons between HC, iRBD-MCI and iRBD+MCI for both neuropsychological and pupillometric variables were run. The Shapiro–Wilk test was used to verify data normality. Based on variables distribution, parametric and non-parametric ANOVAs were applied, and Bonferroni correction was applied for post-hoc pairwise comparisons. In this case, age did not significantly differ between the three groups, thus it was not entered as a covariate in the group comparisons. Moreover, in order to test whether PLR indexes were specifically associated with performances in the visuospatial abilities domain in iRBD patients, non-parametric correlations using Spearman's rho were performed between global visuospatial abilities

scores and PLR parameters.

Finally, two logistic regression analyses using the Backward method were performed. The first objective was to determine whether PLR indexes could predict the category of a subject as either HC or iRBD. The second objective was to determine whether PLR indexes could predict the possible presence of MCI in patients with iRBD.

For all the analyses, p-values less than 0.05 were considered statistically significant.

4.3. Results

4.3.1. Descriptive Statistics

The descriptive statistics of HC and iRBD groups are illustrated in Table 10. A total of 38 iRBD patients (28 males, 10 females) and 18 HC (8 males, 10 females) were included in the study. iRBD patients had a mean age of 66.42 ± 9.62 years, while HC had a mean age of 60.96 ± 8.02 years, which significantly differed between the two groups ($p = 0.018$). Notably, years of education did not differ between the two groups. iRBD patients had a mean disease duration of 4.32 ± 3.01 years, which was computed as the time between subjective symptoms onset and the time of diagnosis.

Descriptives Variables	HC n=18	iRBD n=38
M/F	8/10	28/10
Age, years (mean ± SD, range)	60.96 ± 8.02 (47.00-77.00)	66.42 ± 9.62 (48.00 – 82.00)
Years of education (mean ± SD)	12.00 ± 3.16	12.34 ± 3.59
Disease duration (mean ± SD)	-	4.32 ± 3.01
UPDRS total score (mean ± SD)	-	7.49 ± 5.61
SCOPA total score (mean ± SD)	-	7.82 ± 5.55
NMSQ total score (mean ± SD)	-	26.05 ± 22.71

Table 10. Descriptives statistics of HC and iRBD groups. Abbreviations: F, females; HC, healthy controls; iRBD, isolated Rapid Eye-Movement Sleep Behavior Disorder; M, males; NMSQ, Non-Motor Symptoms Questionnaire; SCOPA, Scale for Outcomes in Parkinson's Disease; SD, standard deviation; UPDRS, Unified Parkinson's Disease Rating Scale.

Based on the cognitive evaluation, the possible presence of MCI was assessed resulting in 18 iRBD-MCI patients (15 males, 3 female) and 20 iRBD+MCI patients (13 males, 7 females). The descriptive statistics of HC, iRBD-MCI and iRBD+MCI groups are illustrated in Table 11. iRBD-MCI patients had a mean age of 65.56 ± 9.19 years, while

iRBD+MCI had a mean age of 67.20 ± 10.17 years. Notably, none of the variables listed in Table 11 differed between the groups.

Descriptives Variables	HC n=18	iRBD-MCI n=18	iRBD+MCI n=20
M/F	8/10	15/3	13/7
Age, years (mean ± SD, range)	60.96 ± 8.02 (47.00-77.00)	65.56 ± 9.19 (52.00-82.00)	67.20 ± 10.17 (48.00-81.00)
Years of education (mean ± SD)	12.00 ± 3.16	12.78 ± 4.04	11.95 ± 3.19
Disease duration (mean ± SD)	-	4.44 ± 2.92	4.20 ± 3.17
UPDRS total score (mean ± SD)	-	6.06 ± 4.84	8.83 ± 6.07
SCOPA total score (mean ± SD)	-	7.39 ± 5.37	8.20 ± 5.82
NMSS total score (mean ± SD)	-	19.56 ± 18.62	31.90 ± 24.85

Table 11. Descriptives statistics of HC, iRBD-MCI and iRBD+MCI groups. Abbreviations: F, females; HC, healthy controls; iRBD, iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; M, males; NMSQ, Non-Motor Symptoms Questionnaire; SCOPA, Scale for Outcomes in Parkinson's Disease; SD, standard deviation; UPDRS, Unified Parkinson's Disease Rating Scale.

4.3.2. Comparisons between HC and iRBD on Neuropsychological data

Considering the entire cohort, iRBD patients showed significantly lower scores on the token test ($p = 0.010$), the copy of the ROCF ($p = 0.003$) and the attentive matrices test ($p = 0.001$) compared to HC. Concerning the VOSP battery, iRBD patients showed significantly lower performances on the silhouettes ($p = 0.002$) and number location ($p = 0.007$) subtests, but higher performances on the object decision subtest ($p = 0.003$) compared to HC. Notably, no significant differences were observed on global cognitive domain scores between HC and iRBD groups.

The cognitive evaluation was also compared between HC, iRBD-MCI and iRBD+MCI groups (see Table 12). iRBD+MCI patients showed significantly lower performances on the token test ($p = 0.004$), the copy ($p < 0.001$) and recall ($p = 0.010$) of the ROCF, the RPM test ($p < 0.001$) and the incomplete letters ($p = 0.009$), the silhouettes ($p < 0.001$), the position discrimination ($p = 0.017$), the number location ($p = 0.002$) and the cube analysis ($p = 0.030$) subtests of the VOSP battery compared to HC. Moreover, iRBD+MCI patients also showed significantly lower performances on the MMSE ($p = 0.028$), the Corsi block-tapping test ($p = < 0.001$), the recall of the ROCF ($p < 0.001$), the RPM test ($p = 0.041$), the TMT A ($p = 0.032$) and the incomplete letters ($p = 0.001$), the silhouettes ($p = 0.005$), the position discrimination ($p = 0.008$) and the cube analysis ($p <$

0.001) of the VOSP battery compared to iRBD-MCI patients.

Neuropsychological tests	Pathological Cut-Offs	HC n=18 (mean ± SD)	iRBD-MCI n=18 (mean ± SD)	iRBD+MCI n=20 (mean ± SD)	p-value (Bonferroni adj)
MMSE	<24.00	28.41 ± 1.16	28.97 ± 0.95	28.02 ± 1.35	1 > 2 (p = 0.028)
Token test	<26.50	33.73 ± 1.56	32.72 ± 1.56	31.59 ± 2.49	0 > 2 (p = 0.004)
Semantic fluency	<25.00	48.38 ± 9.88	50.06 ± 7.46	43.30 ± 10.49	NS
Phonemic fluency	<17.00	38.25 ± 8.20	35.67 ± 11.51	30.50 ± 10.87	NS
Oral naming test	<41.99	47.72 ± 0.73	47.95 ± 0.11	47.32 ± 1.68	NS
Digit forward	<4.26	5.79 ± 0.93	6.21 ± 1.03	5.86 ± 1.01	NS
Digit backward	<2.65	4.22 ± 0.73	4.79 ± 1.06	4.04 ± 1.10	NS
Corsi span test	<3.46	5.10 ± 0.66	5.79 ± 0.76	4.80 ± 0.80	1 > 2 (p < 0.001)
RAVLT-I	<28.53	46.25 ± 12.88	47.18 ± 6.29	42.09 ± 6.84	NS
RAVLT-D	<4.69	10.95 ± 2.57	10.56 ± 2.49	9.26 ± 2.34	NS
RAVLT-R	<8.00	14.06 ± 1.06	14.17 ± 1.25	13.90 ± 1.52	NS
ROCF-C	<28.88	34.85 ± 1.46	33.89 ± 1.63	31.09 ± 3.83	0 > 2 (p < 0.001)
ROCF-R	<9.47	20.23 ± 4.87	22.58 ± 5.20	14.94 ± 5.30	0 > 2 (p = 0.010) 1 > 2 (p < 0.001)
RPM	<18.00	33.34 ± 2.14	31.39 ± 2.64	28.20 ± 5.45	0 > 2 (p < 0.001) 1 > 2 (p = 0.041)
Attentive matrices	<31.00	49.22 ± 4.66	50.71 ± 4.88	48.66 ± 6.47	NS
TMT A	>93	29.00 ± 18.40	20.44 ± 5.99	33.21 ± 16.98	1 < 2 (p = 0.032)
TMT B	>282	73.06 ± 48.52	50.28 ± 28.35	89.11 ± 73.53	NS
TMT B-A	>186	41.25 ± 24.35	30.06 ± 25.23	57.16 ± 60.89	NS
VOSP - Screening Test	Age < 50: > 15 Age ≥ 50: > 15	19.44 ± 0.89	19.17 ± 1.04	18.70 ± 2.42	NS
VOSP - Incomplete letters	Age < 50: > 17 Age ≥ 50: > 16	19.44 ± 0.63	19.56 ± 0.62	17.70 ± 2.62	0 > 2 (p = 0.009) 1 > 2 (p = 0.001)
VOSP - Silhouettes	Age < 50: > 16 Age ≥ 50: > 15	21.75 ± 3.61	19.94 ± 3.26	16.05 ± 3.91	0 > 2 (p < 0.001) 1 > 2 (p = 0.005)
VOSP - Object decision	Age < 50: > 15 Age ≥ 50: > 14	16.75 ± 2.15	18.89 ± 2.17	17.95 ± 3.02	0 < 1 (p = 0.004)
VOSP - Progressive silhouettes	Age < 50: < 14 Age ≥ 50: < 15	7.38 ± 2.78	7.00 ± 2.25	8.65 ± 2.28	NS
VOSP - Dot counting	Age < 50: > 8 Age ≥ 50: > 8	9.88 ± 0.34	9.83 ± 0.51	9.55 ± 0.76	NS
VOSP - Position discrimination	Age < 50: > 18 Age ≥ 50: > 18	19.75 ± 0.78	19.83 ± 0.51	18.90 ± 1.37	0 > 2 (p = 0.017) 1 > 2 (p = 0.008)
VOSP - Number location	Age < 50: > 7 Age ≥ 50: > 7	9.63 ± 0.72	9.11 ± 1.13	7.30 ± 2.70	0 > 2 (p = 0.002)
VOSP - Cube analysis	Age < 50: > 6 Age ≥ 50: > 6	8.69 ± 1.70	9.67 ± 0.49	7.00 ± 1.95	0 > 2 (p = 0.030) 1 > 2 (p < 0.001)

Table 12. Comparisons between HC (0), iRBD-MCI (1) and iRBD+MCI (2) patients on neuropsychological scores. Abbreviations: Bonferroni adj, Bonferroni adjusted; HC, healthy controls; iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; MMSE, Mini-Mental State Examination; NS, not significant; RAVLT-I; Rey auditory verbal learning test – immediate recall; RAVLT-D; Rey auditory verbal learning test – delayed recall; RAVLT-R; Rey auditory verbal learning test – recognition; ROCF-C, copy of the Rey–Osterrieth complex figure; ROCF-R, recall of the Rey–Osterrieth complex figure; RPM, Raven Progressive Matrices test; SD, standard deviation; TMT A, Trail Making Test part A; TMT B, Trail Making Test part B; TMT B-A, Trail Making Test part B-A; VOSP, Visual Object and Space Perception battery.

Concerning global cognitive domains, iRBD+MCI patients showed significantly lower scores in language ($p = 0.025$) and visuospatial abilities ($p < 0.001$) cognitive domains compared to HC. Moreover, iRBD+MCI patients were also more impaired in cognitive screening ($p = 0.027$), memory ($p < 0.001$), executive function ($p = 0.014$) and

visuospatial abilities ($p < 0.001$) cognitive domains compared to iRBD-MCI. Regarding the visuospatial abilities cognitive domain, iRBD+MCI patients performed significantly worse not only in the global domain, but also in both object ($p < 0.02$) and space ($p < 0.001$) perception abilities in comparison to HC and iRBD-MCI (see Table 13).

	HC n=18 (mean ± SD)	iRBD-MCI n=18 (mean ± SD)	iRBD+MCI n=20 (mean ± SD)	p-value (Bonferroni adj)
Visuospatial Abilities Domain	0.26 ± 0.37	0.30 ± 0.17	-0.48 ± 0.58	0 < 1 (NS) 0 > 2 (p < 0.001) 1 > 2 (p < 0.001)
VOSP – Object Perception	0.16 ± 0.57	0.32 ± 0.38	-0.29 ± 0.49	0 < 1 (NS) 0 > 2 (p = 0.019) 1 > 2 (p < 0.001)
VOSP – Space Perception	0.29 ± 0.49	0.36 ± 0.27	-0.56 ± 0.67	0 < 1 (NS) 0 > 2 (p < 0.001) 1 > 2 (p < 0.001)

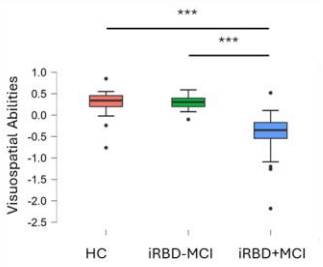


Table 13. The table illustrates the comparisons between HC (0), iRBD-MCI (1) and iRBD+MCI (2) patients on visuospatial abilities global scores. The comparison between the three groups on the visuospatial abilities cognitive domain is also depicted by the graph on the right of the table. * indicate the level of significance. Abbreviations: Bonferroni adj, Bonferroni adjusted; HC, healthy controls; iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; SD, standard deviation; VOSP, Visual Object and Space Perception battery.

4.3.3. Comparisons between HC and iRBD patients on Pupillometric data

Considering the entire cohort, iRBD patients showed a significantly altered PLR compared to HC. Specifically, iRBD patients showed significantly higher variation ($p < 0.001$) and latency ($p = 0.026$), and significantly lower baseline pupil diameter ($p < 0.001$) and VCmax ($p < 0.001$) compared to HC. Notably, when adjusting for age, only the difference in VCmax remained significant ($p = 0.008$) (see Table 14 and Figure 14).

Pupillometric Variables	HC n=18 (mean ± SD)	iRBD n=38 (mean ± SD)	p-value
Base (mm)	5.25 ± 0.90	4.89 ± 0.94	NS
Variation (mm)	-2.40 ± 0.47	-2.22 ± 0.53	NS
Latency (ms)	236.60 ± 42.56	245.92 ± 41.37	NS
Vcmax (mm/s)	4.77 ± 0.92	4.35 ± 0.98	0.008

Table 14. Differences in PLR parameters between HC and iRBD patients. Abbreviations: HC, healthy controls; iRBD, isolated Rapid Eye-Movement Sleep Behavior Disorder; SD, standard deviation; mm, millimeters; ms, milliseconds; mm/s; millimeters per second; NS, not significant.

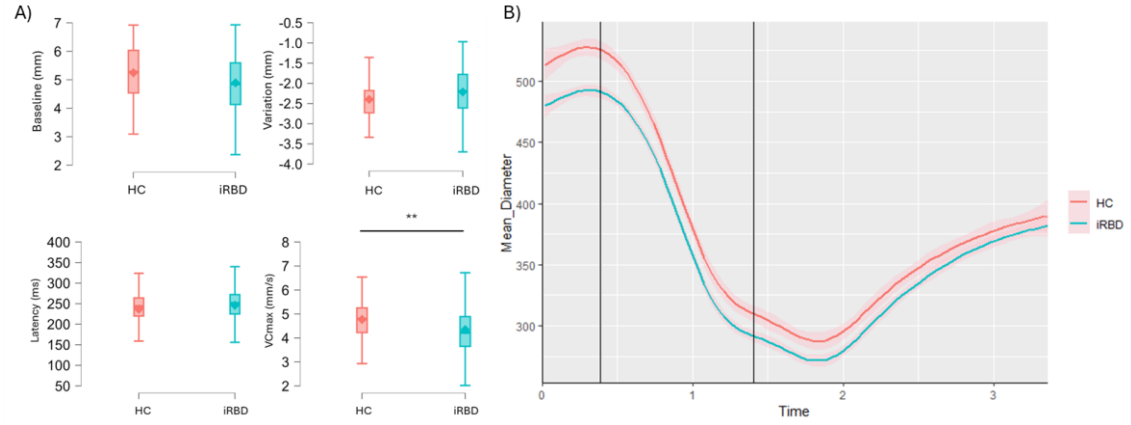


Figure 14. A) Graphical representation of the differences in PLR parameters between HC (red) and iRBD patients (blue). * indicate the level of significance (adjusted for age). **B) Graphical representation of the PLR curves of HC (red) and iRBD patients (blue).** Abbreviations: HC, healthy controls; iRBD, isolated Rapid Eye-Movement Sleep Behavior Disorder; mm, millimeters; ms, milliseconds; mm/s; millimeters per second.

The pupillometric evaluation was also compared between HC, iRBD-MCI and iRBD+MCI groups (see Table 15 and Figure 15). Both iRBD-MCI and iRBD+MCI patients showed significantly higher variation ($p \leq 0.001$) and latency ($p < 0.001$), and significantly lower baseline pupil diameter ($p < 0.05$) and VCmax ($p < 0.001$) compared to HC. Moreover, iRBD+MCI patients also showed a significantly higher latency ($p < 0.001$) and a significantly lower VCmax ($p = 0.035$) compared to iRBD-MCI. Notably, age did not significantly differ between the three groups, thus it was not entered as a covariate in the group comparisons.

Pupillometric Variables	HC n=18 (mean ± SD)	iRBD-MCI n=18 (mean ± SD)	iRBD+MCI n=20 (mean ± SD)	p-value (Bonferroni adj)
Base (mm)	5.25 ± 0.90	4.83 ± 0.87	4.94 ± 1.00	0 > 1 ($p < 0.001$) 0 > 2 ($p = 0.013$) 1 < 2 (NS)
Variation (mm)	-2.40 ± 0.47	-2.18 ± 0.46	-2.25 ± 0.59	0 < 1 ($p < 0.001$) 0 < 2 ($p = 0.001$) 1 < 2 (NS)
Latency (ms)	236.60 ± 42.56	236.32 ± 40.34	254.55 ± 40.47	0 > 1 (NS) 0 < 2 ($p < 0.001$) 1 < 2 ($p < 0.001$)
Vcmax (mm/s)	4.77 ± 0.92	4.47 ± 0.87	4.24 ± 1.05	0 > 1 ($p < 0.001$) 0 > 2 ($p < 0.001$) 1 > 2 ($p = 0.035$)

Table 15. Differences in PLR parameters between HC (0), iRBD-MCI (1) and iRBD+MCI (2). Abbreviations: HC, healthy controls; iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-

Movement Sleep Behavior Disorder with Mild Cognitive Impairment; SD, standard deviation; mm, millimeters; ms, milliseconds; mm/s; millimeters per second; NS, not significant.

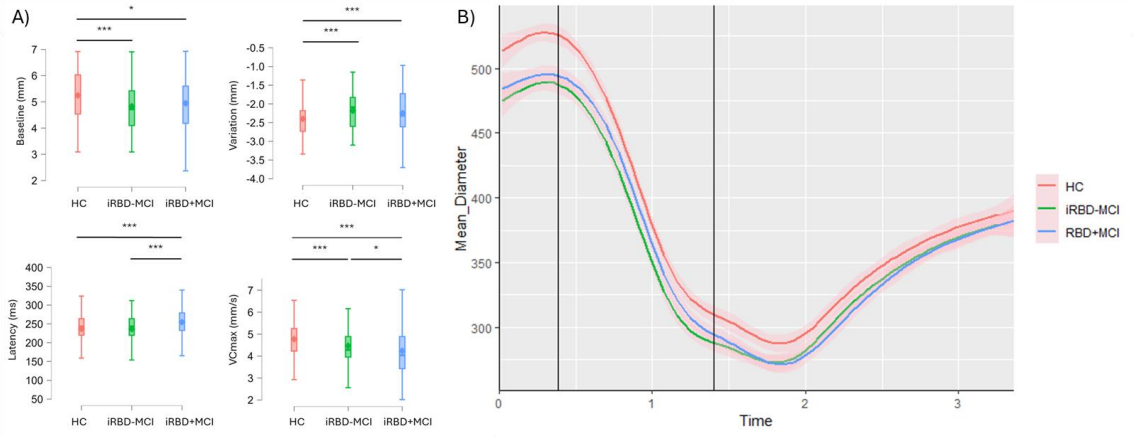


Figure 15. A) Graphical representation of the differences in PLR parameters between HC (red), iRBD-MCI (green) and iRBD+MCI (blue). * indicate the level of significance. **B) Graphical representation of the PLR curves of HC (red), iRBD-MCI (green) and iRBD+MCI (blue).** Abbreviations: HC, healthy controls; iRBD-MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder without Mild Cognitive Impairment; iRBD+MCI, isolated Rapid Eye-Movement Sleep Behavior Disorder with Mild Cognitive Impairment; mm, millimeters; ms, milliseconds; mm/s; millimeters per second.

4.3.4. Association between PLR and Visuospatial Abilities scores in iRBD

The results of the correlation analysis demonstrated an association between PLR indexes and performances in visuospatial abilities in iRBD patients. Specifically, latency showed significant negative correlations with performances in visuospatial abilities cognitive domain ($r = -0.407$; $p = 0.011$) and with object perception scores ($r = -0.494$; $p = 0.002$) (see Figure 16). This means that a longer latency of the pupil reaction to light was associated with lower performances in visuospatial abilities and object perception skills. Moreover, VCmax showed a significant positive correlation with object perception scores ($r = 0.391$; $p = 0.015$) (see Figure 16). That is, a slower VCmax was associated with lower performances in object perception skills. Of note, the correlation between VCmax and performances in visuospatial abilities cognitive domain was close to significance ($r = 0.391$; $p = 0.051$).

No significant correlation has been found between PLR indexes and performances in visuospatial abilities in HC.

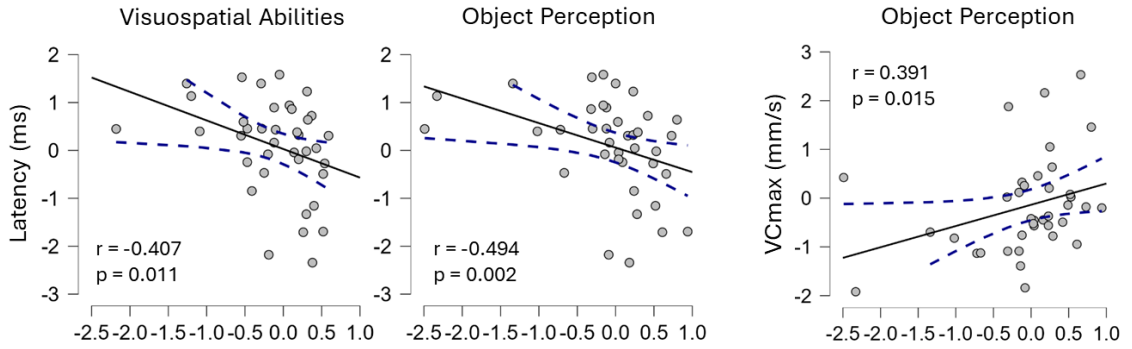


Figure 16. The first scatter plot illustrates the correlation between latency and performances in visuospatial abilities cognitive domain. The second scatter plot illustrates the correlation between latency and object perception scores. The third scatter plot illustrates the correlation between VCmax and object perception scores. Abbreviations: ms, milliseconds; mm/s; millimeters per second; VCmax, maximum constriction velocity.

4.3.5. Logistic Regression Analyses

Two logistic regression analyses using the Backward method were performed. The first objective was to determine whether PLR indexes could predict the category of a subject as either HC or iRBD. In this case, the category (coded as HC = 0, iRBD = 1) was entered as the dependent variable, while the PLR curve indexes (i.e., baseline pupil diameter, variation, latency, VCmax) were entered as covariates. The logistic regression outcome showed that the optimal estimated model was model 0, which demonstrated a significant overall fit (deviance = 665.64; Akaike information criterion (AIC) = 675.74). The results demonstrated that baseline pupil diameter ($p = 0.001$), latency ($p = 0.033$) and VCmax ($p < 0.001$) were statistically significant predictors. The performance metrics of the model revealed an area under the curve (AUC) of 0.683, indicating a moderate level of discrimination between the two categories. Notably, the model exhibited high sensitivity (0.924), suggesting that it accurately identified a large proportion of true positive cases; but it had very low specificity (0.083), indicating a high rate of false positives cases. These findings suggest that the logistic regression model showed a moderate level of effectiveness in predicting the category of a subject (HC or iRBD) based the baseline pupil diameter, latency and VCmax, while also highlighting the need for cautious interpretation of the specificity results (see Table 16).

Model	Deviance	AIC	BIC	df	McFadden R^2	Nagelkerke R^2	Tjur R^2	Cox & Snell R^2
M_0	665.64	675.64	697.28	555	0	0	0.07	0

Model	Parameters	Estimate	Standard Error	z	Wald Statistic	df (Wald test)	p-value (Wald test)
M_0	(Intercept)	2.99	0.85	3.52	12.41	1	$p < 0.001$
	Variation (mm)	-0.62	0.37	-1.68	2.83	1	NS
	Latency (ms)	0.005	0.002	2.13	4.55	1	$p = 0.033$
	Vmax (mm/s)	-0.45	0.13	-3.48	12.13	1	$p < 0.001$
	Base (mm)	-0.55	0.17	-3.20	10.24	1	$p = 0.001$

Performance metrics	Value
AUC	0.683
Sensitivity	0.924
Specificity	0.083

Table 16. Summary output of the logistic regression analysis with Backward method aiming to determine whether PLR indexes could predict the category of a subject as either HC or iRBD. Abbreviations: AIC, Akaike information criterion; AUC, area under the curve; BIC, Bayesian information criterion; df, degrees of freedom; NS, not significant.

The second objective was to determine whether PLR indexes could predict the possible presence of MCI in patients with iRBD. In this case, the presence or absence of MCI (coded as iRBD-MCI = 0, iRBD+MCI = 1) was entered as the dependent variable, while the PLR curve indexes (i.e., baseline pupil diameter, variation, latency, VCmax) were entered as covariates. The logistic regression outcome showed that the optimal estimated model was model 0, which demonstrated a significant overall fit (deviance = 489.73; AIC = 499.73). The results demonstrated that variation ($p = 0.001$), latency ($p < 0.001$) and VCmax ($p < 0.001$) were statistically significant predictors. The performance metrics of the model revealed an AUC of 0.660, indicating a fair ability of the model to discriminate between iRBD-MCI and iRBD+MCI patients. Notably, the model exhibited a moderate sensitivity (0.665) and specificity (0.528). These findings suggest that the logistic regression model showed a moderate level of effectiveness in predicting the presence of MCI in iRBD patients, particularly driven by variation, latency and VCmax indexes (see Table 17).

Model	Deviance	AIC	BIC	df	McFadden R^2	Nagelkerke R^2	Tjur R^2	Cox & Snell R^2
M_0	489.73	499.73	519.43	375	0	0	0.09	0

Model	Parameter	Estimate	Standard Error	z	Wald Statistic	df (Wald test)	p-value (Wald test)
M_0	(Intercept)	-1.93	0.96	-2.01	4.04	1	$p = 0.044$
	Variation (mm)	-1.45	0.44	-3.26	10.65	1	$p = 0.001$
	Latency (ms)	0.012	0.003	4.01	16.08	1	$p < 0.001$
	Vmax (mm/s)	-0.57	0.16	-3.57	12.76	1	$p < 0.001$
	Base (mm)	-0.32	0.21	-1.53	2.35	1	NS

Performance metrics	Value
AUC	0.660
Sensitivity	0.665
Specificity	0.528

Table 17. Summary output of the logistic regression analysis with Backward method aiming to determine whether PLR indexes could predict the possible presence of MCI in patients with iRBD. Abbreviations: AIC, Akaike information criterion; AUC, area under the curve; BIC, Bayesian information criterion; df, degrees of freedom; NS, not significant.

4.4. Discussion

To the best of current knowledge, this is the first study evaluating the PLR in iRBD patients compared to HC using a portable pupillometer. The results showed that iRBD patients had a significantly altered PLR compared to HC, with a significantly slower VCmax. Notably, this alteration was even more pronounced in patients with MCI which showed changes in PLR not only compared to HC, but also to cognitively normal patients. Indeed, iRBD+MCI patients were characterized by significantly higher latency and lower VCmax in comparison to both groups. This means that iRBD+MCI patients needed more time to elicit the pupillary constriction reflex, which was then characterized by a slower maximum velocity in its dynamic. Moreover, both iRBD-MCI and iRBD+MCI patients showed significantly higher variation and latency, and significantly lower baseline pupil diameter and VCmax compared to HC. These results are in line with previous pupillometric literature on AD and PD patients (Fotiou et al., 2009; Stergiou et al., 2009). Indeed, these studies reported a significantly altered PLR in PD-MCI and PD+MCI patients compared to HC, specifically characterized by significantly lower ACmax, VCmax and amplitude of the pupil reaction to light (Stergiou et al., 2009). Moreover, the authors reported that PD+MCI patients were significantly impaired in the same parameters also compared to PD-MCI (Stergiou et al., 2009). These results were further confirmed by a study published by Fotiou and collaborators (Fotiou et al., 2009)

comparing the PLR in HC, probable AD, PD-MCI and PD+MCI patients. The authors observed that probable AD, PD-MCI and PD+MCI patients had a significantly altered PLR compared to HC (Fotiou et al., 2009). Moreover, they also reported that PD+MCI patients showed lower ACmax, VCmax and amplitude compared to PD-MCI (Fotiou et al., 2009). Altogether, these results support the evidence that cognitive impairment is strictly related to cholinergic deficiency, which may play a crucial role in the alteration of PLR in neurodegenerative diseases (Fotiou et al., 2009; Stergiou et al., 2009).

In the present study, cognitive evaluation was also compared between HC, iRBD-MCI and iRBD+MCI patients. As expected, iRBD+MCI patients showed worse performances in several neuropsychological tests compared to both HC and iRBD-MCI patients, namely the recall of the ROCF, the RPM test, the incomplete letters, the silhouettes and the position discrimination of the VOSP battery. Moreover, iRBD+MCI patients were also more impaired in language and visuospatial abilities cognitive domains compared to HC, and in cognitive screening, memory, executive function and visuospatial abilities cognitive domains compared to iRBD-MCI. These results align with previous literature on the neuropsychological profile of iRBD patients reporting highly heterogeneous results (Leitner et al., 2024). Indeed, most cross-sectional studies consistently indicated that memory, executive function and visuospatial abilities are the cognitive domains most affected in iRBD (Ferini–Strambi et al., 2004; Leitner et al., 2024; Massicotte-Marquez et al., 2008; Rolinski, Griffanti, et al., 2016; Rolinski, Zokaei, et al., 2016). In the present study, iRBD+MCI patients showed worst performances in visuospatial abilities cognitive domain compared to both HC and iRBD-MCI patients. Notably, iRBD+MCI patients performed significantly worse not only in the visuospatial abilities global domain, but also in both object and space perception abilities in comparison to both HC and iRBD-MCI. This is in line with previous studies reporting deficits in both visuospatial and visuoperceptive skills in iRBD patients (Cagnin et al., 2013; Chwiszczuk et al., 2017; Fantini et al., 2011; Ferini–Strambi et al., 2004; Marques et al., 2010). Indeed, Marques and collaborators (Marques et al., 2010) observed the presence of a visuoperceptive dysfunction in both iRBD and PD with RBD patients specifically related to object perception. Notably, the authors reported no significant difference in visual perception between iRBD and PD with RBD patients or between HC and PD without RBD, suggesting that this may be a RBD-specific cognitive feature more related to a cholinergic

dysfunction (Marques et al., 2010). In addition, Cagnin and collaborators (Cagnin et al., 2013) reported that the presence of RBD (and visual hallucinations) in DLB patients was associated with poorer performances in the VOSP position discrimination subtest, further supporting a possible cholinergic dysfunction underlying not only visuoperceptive but also visuospatial information processing impairments. The authors hypothesized that pathological changes in brain regions like the dorsal mesopontine nuclei may not only contribute to RBD pathophysiology, but also to the impairment in visuospatial and visuoperceptive processing (Cagnin et al., 2013). Indeed, disruptions in these nuclei, which are also connected to thalamic visual areas, may lead to the altered connectivity in the occipital cortex and to the visual processing deficits considered hallmarks of DLB patients (Cagnin et al., 2013). This is also in line with the malignant PD subtype described by Kehagia and colleagues (Kehagia et al., 2012), which was characterized by a PIGD profile with psychiatric manifestations and by a severe involvement of visuospatial abilities cognitive domain mainly due to a posterior cholinergic dysfunction. Of note, several studies reported that both MCI and autonomic dysfunction represent risk factors for the development of this malignant PD subtype (Fereshtehnejad & Postuma, 2017; Kehagia et al., 2012; Vasconcellos & Pereira, 2015; Y. Xu et al., 2016). Based on this evidence, in the present study the association between PLR alterations (reflecting cholinergic deficiency) and performances in the visuospatial abilities cognitive domain was investigated in iRBD patients. Results showed that, in iRBD patients, a longer latency of the PLR was associated with worse performances in the global visuospatial abilities cognitive domain and in object perception skills. In addition, a slower VCmax was associated with lower performances in object perception abilities. These findings suggest a significant association between PLR alterations, specifically prolonged pupil reaction latency and slower VCmax, and poorer performance in visuospatial and visuoperceptive abilities in iRBD patients. Notably, no such association was observed in HC, suggesting that PLR alterations, reflecting cholinergic deficiency, may be a specific marker of visuospatial and visuoperceptive cognitive decline in iRBD patients. These results provide further evidence of the relationship between cholinergic dysfunction, cognitive impairment and dysautonomia in this population.

Finally, to further corroborate these findings, it was tested not only whether PLR indexes could predict the category of a subject as either HC or iRBD, but also whether

they were able to predict the presence of MCI in iRBD patients. The first logistic regression analysis demonstrated moderate effectiveness in predicting subject category (HC or iRBD) based on baseline pupil diameter, latency, and VCmax, though the specificity of these results requires careful interpretation. Additionally, the second logistic regression analysis showed moderate effectiveness in predicting MCI in iRBD patients, with variation, latency, and VCmax being the most significant predictors. These findings align with previous studies on AD and PD patients, where ACmax and VCmax were identified as the most sensitive indexes of cholinergic deficiency and thus of cognitive decline (Fotiou et al., 2009; Stergiou et al., 2009).

The present study had the advantage of being the first study evaluating the PLR in iRBD patients compared to HC using a portable pupillometer; however, it also presented several limitations. First, we did not evaluate the ACmax index, which has been demonstrated to be one of the most sensitive indexes of cholinergic deficiency in previous pupillometric studies on AD and PD patients (Fotiou et al., 2009; Stergiou et al., 2009). Second, the HC recruited in the present study were not matched for age with iRBD patients that may have played a role as a confounding factor (despite the applied age adjustment). Third, it would have been optimal to include also patients with PD-MCI, PD+MCI and PDD/DLB (with and without RBD) in order to characterize PLR alterations across the entire α-synucleinopathies spectrum. Fourth, the present study had a cross-sectional design, which may limit the broader application of the present findings. Fifth, it was not possible to control for other possible confounding factors. Indeed, even though the pupillometric evaluation was performed in a completely dark and silent room with a comfortable temperature, caffeine and nicotine consumption were not controlled. Moreover, not all subjects were tested at the same time of the day and sleepiness level was not assessed. As reported by Kelbsch and colleagues (Kelbsch et al., 2019), all these variables should be considered under ideal experimental conditions to avoid possible confounding. Finally, despite the fact that the PLR is mainly driven by the autonomic balance between the SANS and the PANS, the contribution of the dopaminergic system should also be taken into account. Indeed, the significant role of DA has been demonstrated in several retinal functions, among which light adaptation and other trophic mechanisms (Giza et al., 2011; Witkovsky, 2004). Within this framework, previous studies on PD patients reported retinal DA deficiency which may contribute to alterations

in the PLR (Archibald et al., 2009; Giza et al., 2011). In line with this evidence, structural changes to the retina have also been reported in iRBD patients and were specifically associated with olfactory and nigrostriatal DA degeneration (Lee et al., 2020). Given these premises, although ophthalmologic disorders were an exclusion criterion for the present study, electroretinograms and visual-evoked potentials should be measured in future pupillometric studies on iRBD patients to fully exclude the impact of retinal disorders on the PLR in these patients.

In conclusion, the results of the present study demonstrated that pupillometry may be a possible supportive tool in the detection of early peripheral cholinergic dysfunction associated with cognitive impairment in iRBD patients. Specifically, the presence of PLR alterations was associated with poorer performances in visuospatial and visuoperceptive processing, further supporting the presence of an underlying cholinergic dysfunction. Thus, it can be hypothesized that together these clinical features (i.e., PLR alterations and visuo-spatial/perceptive deficits) may represent prodromal marker of conversion to a more malignant α-synucleinopathy phenotype. Future studies should investigate the PLR within the entire α-synucleinopathies spectrum in a longitudinal study design in order to test its evolution from the prodromal to the final stages of disease.

5. BEHAVIOR & OSCILLATIONS: DISTORSIONS OF TIME IN iRBD

5.1. Introduction

As outlined in chapter 1, PD can be diagnosed only in the presence of three cardinal motor manifestations: bradykinesia, rest tremor and/or rigidity (Postuma et al., 2015). However, early pathological manifestations of the disorder usually appear years before PD diagnosis via a wide range of non-motor features which worsen with the progression of the disease (Poewe et al., 2017). This complex spectrum of motor and non-motor symptoms not only reflect the early and progressive neurodegeneration of DA neurons in the SNpc (Caminiti et al., 2017), but also dysfunctions linked to the extensive connectivity of the basal ganglia. Indeed, basal ganglia are structurally and functionally connected with several deep and surface brain structures overseeing different functions, among which temporal processing (Postuma & Dagher, 2006; Schwartze et al., 2011; Schwartze & Kotz, 2016). Temporal processing is defined as unravelling of time-related cues or producing motor responses with precise timing and it is associated with a broad range of cognitive functions (Foster et al., 2013; Mauk & Buonomano, 2004). Strictly linked to this concept, sensorimotor synchronization relies on temporal processing in order to align movements with external rhythms; indeed, "keep in time" requires the anticipation and prediction of future events (Iversen & Balasubramaniam, 2016; Repp, 2005; Schwartze, Keller, et al., 2011). Recent studies showed that both temporal processing and sensorimotor synchronization can be ascribed to a tangled "timing network" involving not only the basal ganglia and linked cortico-striato-thalamo-cortical circuits, but also the cerebellum, the thalamus, the supplementary motor area (SMA) and the PFC (Schwartze et al., 2011; Schwartze & Kotz, 2013, 2016). On one hand, neuropsychological, neuroimaging, and neuromodulation studies demonstrated that the cerebellum plays a key role both in sensorimotor and perceptual timing on the timescale of tens-to-hundreds of ms – pre-attentive processes (Schwartze et al., 2011; Spencer & Ivry, 2021). Indeed, it is involved not only in the automatic event-based timing of discrete, salient events which allows a timed coordination, but also in online predictive control and error correction, thus ensuring synchronization by anticipating the timing of future events and adjusting to it (Schwartze & Kotz, 2016; Spencer & Ivry, 2021). On the other hand,

basal ganglia and linked cortico-striato-thalamo-cortical circuits are mainly involved in interval-based temporal processing and in the regulation of sensory-motor timing on the timescale of seconds, which is crucial for all movement-related behaviors – attention-dependent processes (Kotz et al., 2016; Mauk & Buonomano, 2004; Schwartze et al., 2011; Schwartze & Kotz, 2016). One of the most important relay stations connected with the basal ganglia system is the thalamus which conveys time-related information to the cortex (Kotz & Schwartze, 2011; Schwartze & Kotz, 2013). Notably, the thalamus performs this important task by orchestrating two distinct performances: the first is by assuming a tonic firing mode which is essential for preserving input linear representation to cortical area; the second is by assuming a burst firing mode, which is instead crucial for a better input discernibility (Kotz & Schwartze, 2011; Schwartze & Kotz, 2013). Thus, the thalamus is pivotal in warning the cortex about modifications happening in the surrounding environment by triggering time-related oscillatory activity (Kotz & Schwartze, 2011). Several studies reported that also higher cortical structures are involved in this tangled "timing network", such as the SMA and related connections (Kotz et al., 2016; Kotz & Schwartze, 2011; Schwartze et al., 2011; Schwartze & Kotz, 2013, 2016). In more detail, the SMA can be structurally (and functionally) partitioning into: 1) the (rostral) pre-SMA which is mainly involved in attention-dependent temporal processing and in the coding of temporal structures both in time perception and production; 2) the (caudal) SMA-proper which is mainly involved in time production related to motor execution by supporting a consistent timing of movements into a sequential action (Kotz & Schwartze, 2011). Finally, also the PFC (and more specifically the lateral PFC) is entangled within this complex "timing network". Back in 1995, Fuster (Fuster, 1995) already demonstrated that the PFC plays a key role in organizing and interpreting time-related information not only by maintaining and manipulating temporal sequences in the working memory, but also by establishing a preparatory set through the prediction of the timing of future events. Indeed, the PFC stands at the highest level of the perception-action cycle by merging them into the brain's representation of the time dimension in order to facilitate goal-directed behaviors (Fuster, 2001). Given these premises, efficient temporal processing and sensorimotor synchronization abilities require that all the components of the "timing network" and their connections are well-preserved (see Figure 17) (Kotz & Schwartze, 2011; Schwartze & Kotz, 2013, 2024).

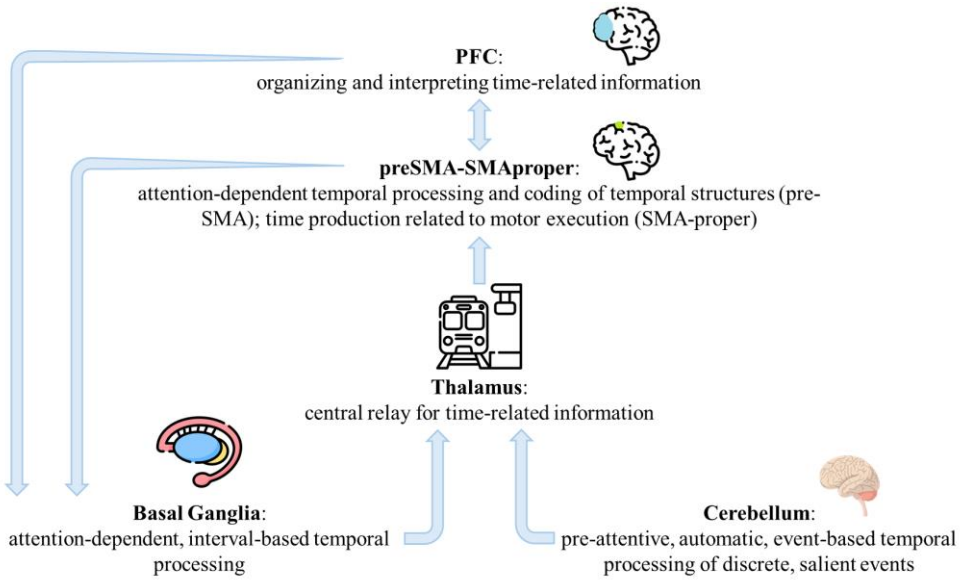


Figure 17. Schematic representation of the "timing network" (simplified). This figure was created by me based on Kotz & Schwartze, 2011 and Schwartze & Kotz, 2013, 2024. All icons are from Flaticon (www.flaticon.com): basal ganglia icon created by Freepik – Flaticon (https://www.flaticon.com/free-icons/basal-ganglia" title="basal ganglia icons); cerebellum icon created by Nsit – Flaticon (https://www.flaticon.com/free-icons/cerebellum" title="cerebellum icons); train station icon created by Freepik – Flaticon (https://www.flaticon.com/free-icons/train-station" title="train station icons); brain icons created by Freepik – Flaticon (https://www.flaticon.com/free-icons/brain" title="brain icons). Abbreviations: PFC, prefrontal cortex; SMA, supplementary motor area.

In this context, several studies on PD patients observed pathological changes to the same components part of the "timing network". Although it is well-known that DA neurodegeneration in the SNpc is the main cause of PD motor symptoms (Caminiti et al., 2017), extensive pathological changes have been reported also in basal ganglia related connections with altered oscillatory activity and synchronization in almost all nuclei (Galvan et al., 2015; Obeso et al., 2008). These disease-related alterations produce a cascading effect on basal ganglia linked cortico-striato-thalamo-cortical circuits (Casarotto et al., 2019; Galvan et al., 2015), reflecting in structural changes and aberrant activity in the thalamus (Halliday, 2009; Salsone et al., 2014), cerebellar atrophy and altered cerebellar activity (Lewis et al., 2013; Li et al., 2023; Ma et al., 2018), and dysfunctions in higher order regions like SMA and PFC (Casarotto et al., 2019; Jia et al., 2022; Kikuchi et al., 2001; Rahimpour et al., 2021; Xing et al., 2024). In this context, different studies suggested that these pathological changes may manifest in the dysfunctional temporal processing and sensorimotor synchronization abilities seen in PD

patients (Allman & Meck, 2012; Dalla Bella et al., 2015; Elsinger et al., 2003; Kotz & Schwartze, 2011; Marinho et al., 2018; Rahimpour et al., 2021; Schwartze & Kotz, 2016). Indeed, timing dysfunctions in PD are clinically exemplified by the wide spectrum of motor-symptoms, among these, bradykinesia and disturbances of gait fluency (e.g., FoG and gait festination) are the main impairments contributing to problems in both synchronization and spontaneous motor tapping (SMT) tasks (Dalla Bella et al., 2015; Schwartze & Kotz, 2016). Despite this, it is currently unknown at which point in the progression of the neurodegenerative process temporal processing and sensorimotor synchronization deficits first appear. To the best of the current knowledge, only one study in the literature investigated the presence of rhythmic dysfunctions in the prodromal stage of PD, that is iRBD (Cochen De Cock et al., 2020). Indeed, Cochen De Cock and colleagues (Cochen De Cock et al., 2020) observed that iRBD patients showed poor performances in both time perception and production tasks compared to HC and that deficits in time production correlated with other PD early markers (i.e., dysautonomia, non-motor deficits, DAT uptake).

Given these premises, the main aim of the present pilot study was to evaluate whether temporal processing and sensorimotor synchronization dysfunctions may be already present in iRBD patients and to investigate EEG-related rhythms.

5.2. Methods

5.2.1. Participants

Twelve HC were recruited at the University of Maastricht, the Netherlands[7]. All subjects were recorded with a 128-channel EEG setup during resting-state EEG and performed the behavioral tasks. Subjects included were adults (> 18 years of age) with no diagnosed hearing impairment and no history of diagnosed neuropsychological disorders.

Moreover, twelve iRBD patients were recruited at the Sleep Disorder Centre of the San Raffaele Hospital, Milan, Italy. The diagnosis of iRBD was confirmed through PSG by a sleep-medicine expert based on the third edition of the ICSD (American Academy

[7] I recruited and tested HC during my period abroad at the Basic & Applied NeuroDynamics Laboratory (BAND Lab), University of Maastricht, the Netherlands. All subjects provided informed written consent and received a compensatory fee. The study was approved by the ethics committee of the University of Maastricht.

of Sleep Medicine, 2014). iRBD patients underwent a comprehensive clinical and neurological evaluation in order to exclude patients with dementia, parkinsonian signs, psychiatric or other neurological disorders (American Psychiatric Association, 2013; Postuma et al., 2015). Eleven out of 12 iRBD patients performed all the behavioral tasks, except one that only performed the SMT and the anisochrony detection with tones tasks.

For both HC and iRBD patients, information regarding handedness and previous musical background was collected.

5.2.2. EEG set-up, pre- and post- processing for HC

To measure brain electrical activity in HC, an elastic actiCAP snap with 128 gel-based active electrodes and actiCHamp Plus operational amplifier (actiCHamp Plus, Brain Products GmbH, Gilching, Germany) were used. Data was acquired using Brain Vision Recorder software (BrainVision Recorder, Brain Products GmbH, Gilching, Germany) with a sampling rate of 1000Hz. Electrode impedance was kept below 10 kΩ.

The pre-processing of EEG data was carried out using Letswave 6 software toolbox (https://github.com/NOCIONS/ Letswave6) for MATLAB (version 9.11, R2021b). Raw EEG signals were first imported and visually inspected for major artifacts. Data was frequency filtered using a high-pass filter of 0.5 Hz and a low-pass filter of 20 Hz. Independent component analysis (ICA) using the probabilistic ICA (PICA) method (Rajan & Rayner, RRN) was applied and components with major artifacts were removed. Finally, artifact rejection using an amplitude criterion of ±100 μV was also conducted. The data were then segmented into 60 s epochs for further analysis and the first segment was removed, following standard procedures for resting-state EEG pre-processing.

Regarding post-processing, after epochs averaging, fast Fourier transform (FFT) was computed, and power was read out within a meaningful time window of ± 0.3 Hz defined around a frequency of interest. In more detail, we wanted to test whether SMT pre tasks rhythms were related to the power of the rhythms produced during resting-state EEG; thus, the ITIs of the SMT pre-tasks (ms) were converted into Hz. This individual's "behavioral frequency" was considered as the frequency of interest for the power analysis. Notably, one frontal-central-parietal region of interest (ROI) was selected (F3, F1, Fz, F2, F4, FFC5h, FFC3h, FFC1h, FFC2h, FFC4h, FFC6h, FC5, FC3, FC1, FC2, FC4, FC6, FCC5h, FCC3h, FCC1h, FCC2h, FCC4h, FCC6h, C5, C3, C1, Cz, C2, C4,

C6, CCP5h, CCP3h, CCP1h, CCP2h, CCP4h, CCP6h, CP5, CP3, CP1, CPz, CP2, CP4, CP6, CPP5h, CPP3h, CPP1h, CPP2h, CPP4h, CPP6h, P3, P1, Pz, P2, P4, PPO1h, PPO2h, POz).

5.2.3. Behavioral tasks and related indexes

The behavioral tasks were based on the 2011 study of Schwartze and colleagues (Schwartze et al., 2011) and consisted in a computerized version of three selected subtests of the battery for the assessment of auditory sensorimotor and timing abilities (BAASTA) (Dalla Bella et al., 2017). BAASTA is a tablet-based application for the evaluation of perceptual and sensorimotor timing skills. The total duration of the battery is 120 min, and it includes four time perception tasks and four time production tasks (Dalla Bella et al., 2017). Computer-based adaptations of the unpaced tapping task (time production), the anisochrony detection with tones task (time perception) and the adaptive tapping task (time production) were created and administered using Presentation® software (version 23.0, Neurobehavioral Systems, Inc., Berkeley, CA, www.neurobs. com) running on a Windows PC. Auditory stimuli (tone frequency: 1047 Hz, duration: 150 ms) were created using Audacity software (https://www.audacityteam.org/) and were presented at a comfortable volume via speakers to HC and using headphones to iRBD patients (JBL Tune 720BT).

In the SMT (or unpaced tapping) task, the subject was asked to tap on the spacebar key with a finger of the right/left hand, trying to maintain the same speed during the whole task. It was performed four times: two times at the beginning – SMT pre (one with the right and one with the left hand), and two times at the end – SMT post (one with the right and one with the left hand) of the session. The duration of each task was 1 min. The aim of the task was to assess the spontaneous tapping rate (and variability) of the subject (Dalla Bella et al., 2017). For each subject, first and last taps were discarded, then outliers were identified and removed using the interquartile range (IQR) method (< Q1-3*IQR, > Q3+3*IQR). Mean inter-tap-interval (ITI), SD, and coefficient of variation (CV)[8] for both SMT pre and post were computed separately for the right and left hands, and averaged for each subject. Notably, right and left conditions were counterbalanced between

[8] Coefficient of variation (CV): $\frac{SD\ of\ individual\ ITI}{mean\ of\ individual\ ITI}$

participants, both for HC and iRBD patients. Finally, the absolute difference (delta) between SMT pre and SMT post tasks was also computed.

In the anisochrony detection with tones task, the subject was asked to judge whether a sequence of five time-consistent sounds contained an irregularity. The duration of the task was 8 min with a total of 40 trials. In regular sequences (20 trials), the inter-onset-interval (IOI) was 600 ms (with an inter-stimulus-interval (ISI) of 450 ms); meanwhile, in irregular sequences (20 trials) the IOI time shift was 180 ms. See Figure 18 for a schematic representation of the task. The aim of the task was to test whether the subject perceives (or not) the presence of a time shift (Dalla Bella et al., 2017). For each subject, reaction times (RTs), SD, and CV were computed; meanwhile, outliers were identified and removed using the IQR method (< Q1-3*IQR, > Q3+3*IQR). Finally, the d-prime (d') index (Swets, 1960) was computed as the difference between the (z-scored) hit[9] rate and (z-scored) false alarm (FA)[10] rate.

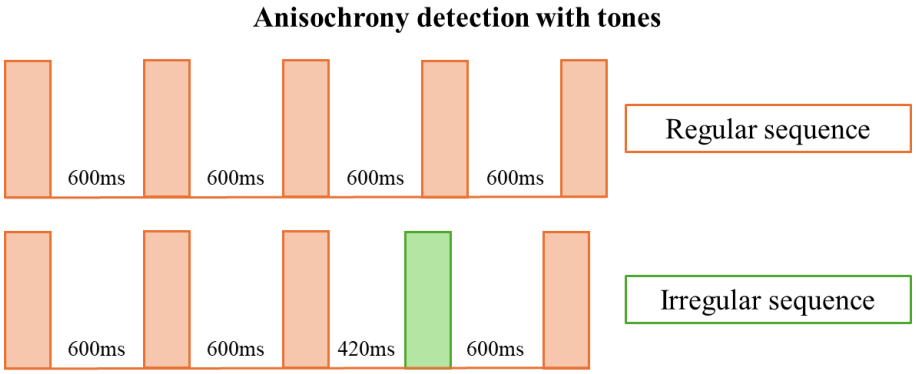


Figure 18. Example of regular and irregular sequences presented during the anisochrony detection with tones task. Abbreviations: ms, milliseconds.

In the adaptive tapping task, the subject was asked to: i) to synchronize with a sequence of five time-consistent sounds; ii) to adapt to eventual time shifts; and iii) to maintain the same rhythm (both with and without time shift) even in the absence of sounds (for 4500

[9] Hits were defined as the correct identification of irregular sequences (both acceleration/ deceleration conditions). The hit rate was computed as: $\frac{n\ of\ hits}{n\ of\ irregular\ sequences}$

[10] False alarms (FA) were defined as the wrong identification of regular sequences as irregular (both acceleration/deceleration conditions). The FA rate was computed as: $\frac{n\ of\ FA}{n\ of\ regular\ sequences}$

ms). The duration of the task was 25 min with a total of 60 trials. In regular sequences (no change condition), the IOI was 600 ms (with an ISI of 450 ms). In slower irregular sequences (deceleration condition) the IOI time shift was 630 ms (p30 condition – 10 trials) or 675 ms (p75 condition – 10 trials); meanwhile, in faster irregular sequences (acceleration condition) the IOI was 570 ms (m30 condition – 10 trials) or 525 ms (m75 condition – 10 trials). Notably, the task was preceded by a 2 min training. After each trial, the subject was asked whether she/he perceived a time shift or not and the direction of the time shift (i.e., acceleration, deceleration or no change). See Figure 19 for a schematic representation of the task. The aim of the task was not only to test the flexibility of a subject in adapting to a time shift and to maintain the new rhythm, but also to test whether the subject perceived (or not) the presence of a time shift (Dalla Bella et al., 2017). Mean ITI, SD, and CV were computed separately for each condition (acceleration, deceleration or no change). Finally, the d' index (Swets, 1960) was also computed as described above.

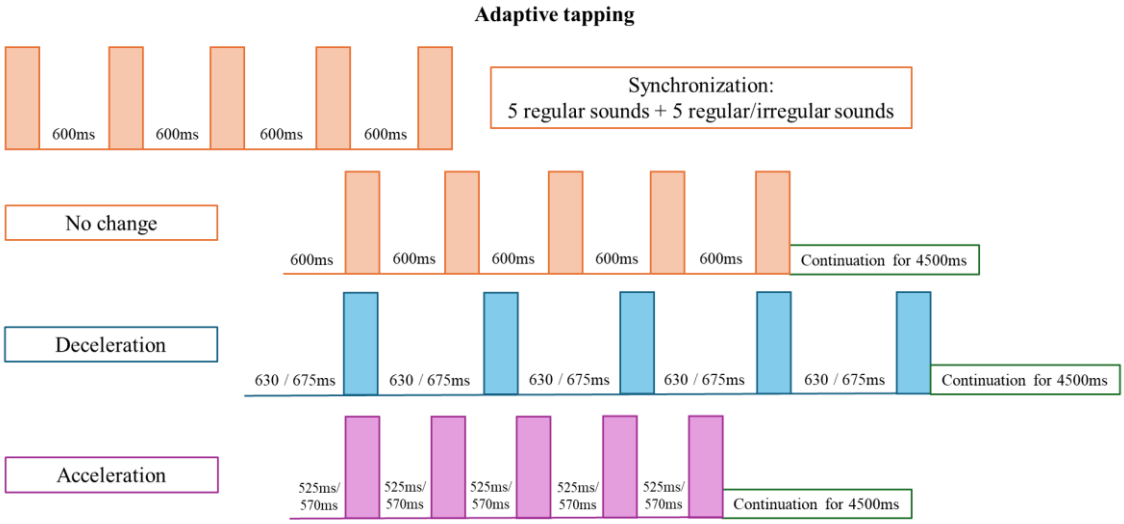


Figure 19. Schematic representation of the adaptive tapping task. Concerning the synchronization phase: orange rectangles refer to the five initial regular sound and to the five sounds of the "no change condition"; blue rectangles refer to the five sounds of the "deceleration condition"; pink rectangles refer to the five sounds of the "acceleration condition". Finally, dark green rectangles refer to the continuation phase. Abbreviations: ms, milliseconds.

In addition to these tasks, also the preferred perceptual tempo (PPT) was assessed using the Asparion Metronomo+ software (version 3.0.4., 2021-2015, for Windows). In the PPT, the subject was asked to play around with the metronome and to choose her/his

preferred tempo. The starting rate of the metronome could be 80 beats per minute (bpm) or 120 bpm and it was counterbalanced between participants, both for HC and iRBD patients. The PPT is defined as a zone of optimal tempo, at which people are best able to process events and seems to be centered around 600 ms IOI (range 300-800 ms IOI) (Baruch et al., 2004; Fraisse, 1963). Notably, previous studies demonstrated that the PPT was correlated with the SMT (McAuley et al., 2006).

5.2.4. Experimental design

Concerning HC, the experimental design consisted of a single session of 2 h, including 60 min of preparation time and 60 min of testing time. After 15 min of eye-closed resting-state EEG, the different behavioral tasks were administered: SMT right and left pre-tasks, PPT, anisochrony detection with tones task, adaptive tapping task and SMT right and left post-tasks. The experimental design was the same for iRBD patients except that resting-state EEG was not recorded, consequently a single session lasted 45 min for patients. Figure 20 illustrates the experimental design of the study.

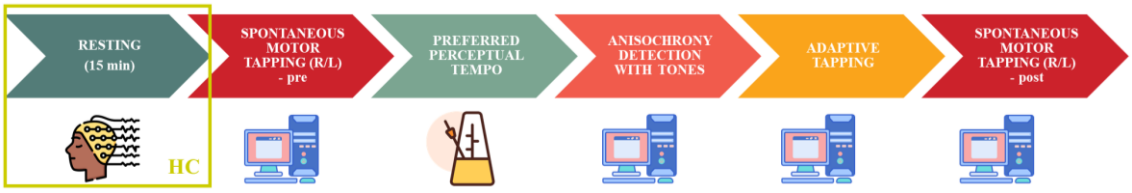


Figure 20. Experimental design of the study. This figure was created by me. All icons are from Flaticon (www.flaticon.com): EEG icons created by Freepik – Flaticon (https://www.flaticon.com/free-icons/eeg" title="eeg icons); computer icons created by Flat Icons – Flaticon (https://www.flaticon.com/free-icons/computer" title="computer icons); rythm (i.e., metronome) icon created by Darius Dan – Flaticon (https://www.flaticon.com/free-icons/rythm" title="rythm). Abbreviation: HC, healthy controls; L, left; R, right.

5.2.5. Statistical Analyses

The data analysis was performed using JASP (version 0.19.1). Descriptive statistics were run for both groups (HC and iRBD) and data regarding age, gender, handedness, and previous musical background were collected. For all the variables included in the following analyses, the Shapiro–Wilk test was used to verify data normality and, based on variables distribution, parametric or non-parametric tests were applied.

Concerning the SMT task, in order to test if there were differences between right and

left SMT performances, comparisons on handedness in SMT pre and post tasks for both HC and iRBD were run using paired samples t-tests. Then, HC and iRBD patients were compared in terms of SMT ITI, SD and CV (right/left separately and averaged pre/post) using non-parametric ANOVA adjusting for age. In order to test whether SMT performances significantly differed between pre and post tasks, comparisons were performed separately for both HC and iRBD patients using paired sample t-tests. Finally, the absolute differences between SMT pre and SMT post tasks were compared between the two groups using the Mann-Whitney U test. Regarding the anisochrony detection with tones task, the two groups were compared using non-parametric ANOVA adjusting for age. As regards the adaptive tapping task, in order to test if there were differences between HC and iRBD patients in terms of ITI, SD and CV, the two groups were compared using non-parametric ANOVA adjusting for age. Using paired sample t-tests, the synchronization and continuation phases were compared within each group to test whether the subjects were able to adapt and to maintain the rhythm. Then, correlations between the average SMT pre/post tasks and performances on the adaptive tapping task were run to check whether the individual rhythm of the subjects changed in relation to the rhythms of the previously performed tasks. Based on variables distribution, parametric (Pearson's r) or non-parametric (Spearman's rho) correlations were performed. Finally, Spearman's rho was used for the correlation analysis between SMT behavioral pre-task and resting-state EEG data. For all the analyses, p-values less than 0.05 were considered statistically significant.

5.3. Results

5.3.1. Sample characteristics

Twelve iRBD patients (11 males, 1 female) and 12 HC (8 males, 4 females) were included in the study. iRBD patients had a mean age of 64.33 ± 10.65 years (range 49-84), while HC had a mean age of 66.42 ± 9.62 years (range 22-77), which significantly differed between the two groups ($p < 0.001$). Regarding handedness, 83.33 % of HC (10 out of 12) and 75 % of iRBD patients (9 out of 12) were right-handed. None of the subjects had a previous musical background. The mean PPT of HC was 95.14 ± 24.45 bpm (i.e., 632 ms); meanwhile, for iRBD patients was centered around 116.25 ± 26.38 bpm (i.e., 517 ms).

5.3.2. Spontaneous motor tapping tasks

Concerning SMT tasks, no difference between SMT R and SMT L (both pre and post) were observed in either the HC group or the iRBD group; thus, it was reasonable to compute averaged (R-L) ITIs, SD and CV pre and post tasks for both groups. For SMT pre tasks, iRBD patients showed a mean averaged ITI of 755.85 ± 46.23 ms; meanwhile, HC had a mean averaged ITI of 942.33 ± 61.62 ms. For SMT post tasks, iRBD patients showed a mean averaged ITI of 617.40 ± 38.10 ms; meanwhile, HC had a mean averaged ITI of 620.37 ± 34.85 ms. Notably, when comparing averaged (and separated for R-L) ITIs, SD and CV of SMT pre and post tasks between HC and iRBD groups, no significant differences were observed both with and without age adjustment. However, significant differences were found within both groups when comparing ITIs, SD and CV between SMT pre and SMT post tasks (see Table 18). Specifically, ITIs between SMT pre and post showed a significant reduction which was centered around 600ms for both groups.

SMT – HC group	PRE	POST	p-value
Average (R-L) ITI	942.33	620.37	$p = 0.007^b$
Average (R-L) SD	61.62	34.85	$p < 0.001^b$
Average (R-L) CV	0.06	0.05	$p = 0.011^a$
SMT – iRBD group	**PRE**	**POST**	**p-value**
Average (R-L) ITI	755.85	617.40	$p = 0.003^b$
Average (R-L) SD	46.23	38.10	$p = 0.024^a$
Average (R-L) CV	0.06	0.06	NS

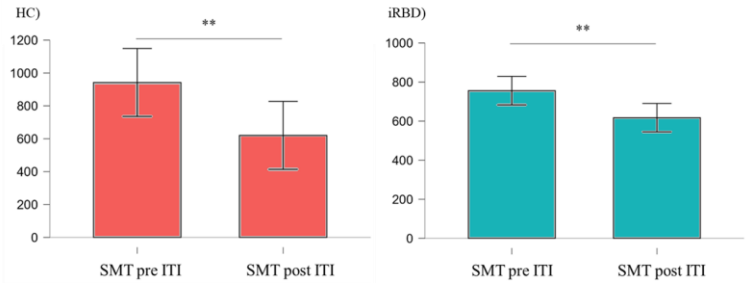


Table 18. Within group comparisons between SMT pre and post tasks for both HC (red graph) and iRBD (blue graph). [a]Student's t-test; [b]Wilcoxon signed-rank test. Abbreviations: CV, coefficient of variation; HC, healthy controls; iRBD, isolated rapid eye-movement sleep behavior disorder; ITI, inter-tap intervals; L, left; NS, not significant; R, right; SD, standard deviation; SMT, spontaneous motor tapping.

In order to test whether the magnitude of the difference between SMT pre and post task differed between the two groups, the absolute difference (delta) between average ITI of SMT pre and SMT post tasks was computed for each subject and each group separately.

However, comparisons using the Mann-Whitney U test revealed no significant differences between the two groups. Finally, no significant correlations between the PPT and the SMT pre tasks were observed.

5.3.3. Anisochrony detection with tones task

Regarding the anisochrony detection with tones task, iRBD patients showed a mean RT of 886.16 ± 338.40 ms with a mean CV of 0.40. Meanwhile, HC had a mean RT of 670.27 ± 230.05 ms with a mean CV of 0.35. However, when comparing the two groups, no significant differences were found both with and without age adjustment.

Finally, d' index was computed and compared between the two groups; however, no significant differences was observed. Figure 21 illustrates the mean number of hits, correct rejections, false alarms and misses of the two groups.

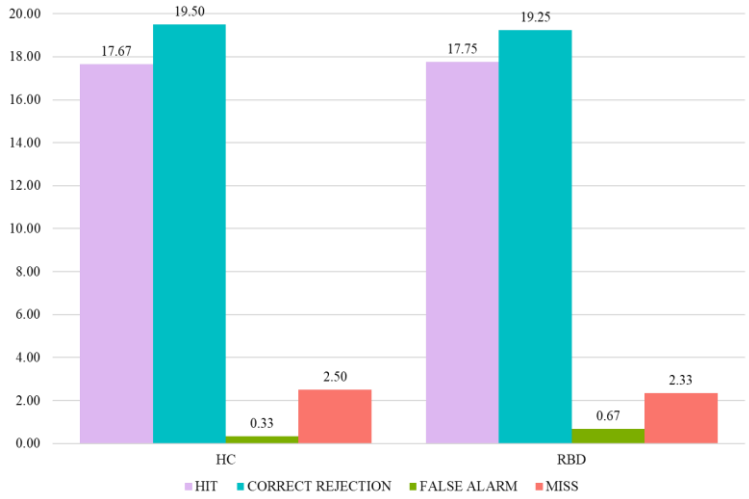


Figure 21. Graphical representation of the mean number of hits (pink), correct rejections (blue), false alarms (green) and misses (red) of HC (on the left) and iRBD patients (on the right) in the anisochrony detection with tones task. Abbreviations: HC, healthy controls; iRBD, isolated rapid eye-movement sleep behavior disorder.

5.3.4. Adaptive tapping task

Concerning the synchronization part of the adaptive tapping task, iRBD patients showed mean ITI for regular sequences (no change condition) of 551.86 ± 69.54 ms which significantly differed from the mean ITI for regular sequences of the continuation part ($p = 0.009$). For faster irregular sequences, iRBD patients showed a mean ITI of 553.54 ± 30.72 ms and of 497.96 ± 39.21 ms for 570 ms (m30) and 525 ms (m75)

conditions respectively. For slower irregular sequences, iRBD patients had a mean ITI of 625.44 ± 37.69 ms and of 664.85 ± 47.42 ms for 630 ms (p30) and 675 ms (p75) conditions respectively. For both acceleration (m30 and m75) and deceleration (p30 and p75) conditions, iRBD patients showed no significant differences between synchronization and continuation phases performances. Regarding variability, highest CV was reported for the no change condition of the continuation phase, which also significantly differed from the mean CV of the synchronization phase ($p < 0.001$). Moreover, variability was significantly higher also for the m30 condition of the continuation phase compared to the synchronization phase ($p = 0.005$). Table 19 illustrates within group comparisons between synchronization and continuation phases of the adaptive tapping task for the iRBD group.

Adaptive tapping task – iRBD group	SYNC	CONT	p-value
ITI no change (mean ± SD)	551.86 ± 69.54	582.07 ± 25.76	p = 0.009[a]
ITI m30 (mean ± SD)	553.54 ± 30.72	546.97 ± 20.75	NS
ITI m75 (mean ± SD)	497.96 ± 39.21	507.24 ± 36.92	NS
ITI p30 (mean ± SD)	625.44 ± 37.69	613.70 ± 29.14	NS
ITI p75 (mean ± SD)	664.85 ± 47.42	658.49 ± 30.15	NS

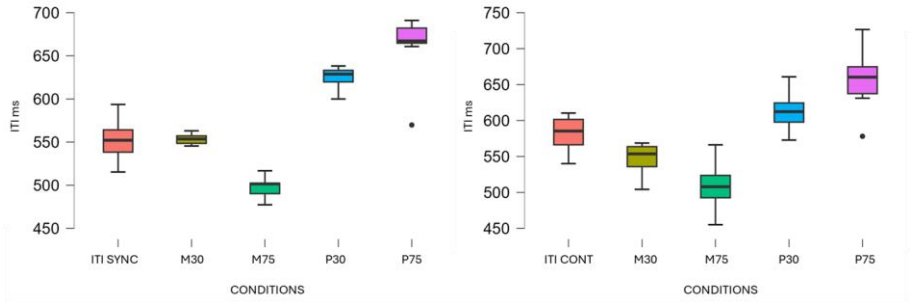


Table 19. Within group comparisons between synchronization and continuation phases of the adaptive tapping task for the iRBD group. [a]Student's t-test. Abbreviations: CONT, continuation; CV, coefficient of variation; HC, healthy controls; iRBD, isolated rapid eye-movement sleep behavior disorder; ITI, inter-tap intervals; m30, minus 30 milliseconds; m75, minus 75 milliseconds; NS, not significant; p30, plus 30 milliseconds; p75, plus 75 milliseconds; SD, standard deviation; SYNC, synchronization.

With respect to the synchronization part of the adaptive tapping task, HC showed mean ITI for regular sequences (no change condition) of 555.90 ± 57.87 ms which significantly differed from the mean ITI for regular sequences of the continuation part ($p = 0.045$). For faster irregular sequences, HC showed a mean ITI of 553.20 ± 31.44 ms and of 490.22 ± 36.18 ms for 570 ms (m30) and 525 ms (m75) conditions respectively. For slower

irregular sequences, HC had a mean ITI of 621.64 ± 36.52 ms and of 678.87 ± 43.12 ms for 630 ms (p30) and 675 ms (p75) conditions respectively. For both acceleration (m30 and m75) and deceleration (p30 and p75) conditions, HC patients showed no significant differences between synchronization and continuation phases performances. Regarding variability, highest CV was reported for the no change condition of the synchronization phase, which was also significantly different from the mean CV of the continuation phase ($p < 0.001$). Moreover, variability was significantly higher also for the p30 and m75 conditions of the synchronization compared to the continuation phase ($p = 0.010$). Table 20 illustrates within group comparisons between synchronization and continuation phases of the adaptive tapping task for the HC group.

Adaptive tapping task – HC group	SYNC	CONT	p-value
ITI no change (mean ± SD)	555.90 ± 57.87	583.33 ± 19.57	p = 0.045[a]
ITI m30 (mean ± SD)	553.20 ± 31.44	547.21 ± 31.44	NS
ITI m75 (mean ± SD)	490.22 ± 36.18	490.31 ± 36.19	NS
ITI p30 (mean ± SD)	621.64 ± 36.52	618.09 ± 36.52	NS
ITI p75 (mean ± SD)	678.87 ± 43.12	688.92 ± 43.12	NS

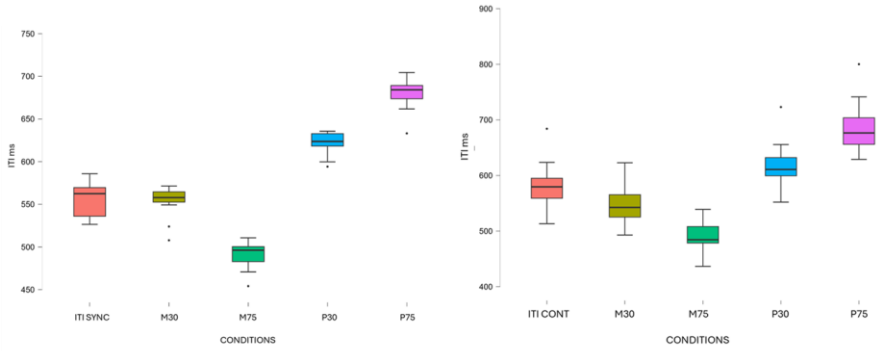


Table 20. Within group comparisons between synchronization and continuation phases of the adaptive tapping task for the HC group. [a]Student's t-test. Abbreviations: CONT, continuation; CV, coefficient of variation; HC, healthy controls; iRBD, isolated rapid eye-movement sleep behavior disorder; ITI, inter-tap intervals; m30, minus 30 milliseconds; m75, minus 75 milliseconds; NS, not significant; p30, plus 30 milliseconds; p75, plus 75 milliseconds; SD, standard deviation; SYNC, synchronization.

When comparing synchronization and continuation performances (for all conditions) between HC and iRBD patients, no significant differences were observed.

Notably, correlations between synchronization and continuation performances and averaged ITI of SMT post tasks were performed separately for each group. In the iRBD group, averaged ITI of SMT post tasks was significantly inversely associated with mean

ITI of no change condition in the synchronization phase (r = -0.626, p = 0.039). In the HC group, averaged ITI of SMT post tasks was significantly positively associated with mean ITI of no change condition in both synchronization (r = 0.671, p = 0.02) and continuation (r = 0.727, p = 0.01) phases.

Finally, d' index was computed and compared between the two groups; however, no significant differences was observed. Figure 22 illustrates the mean number of hits, correct rejections, false alarms and misses of the two groups.

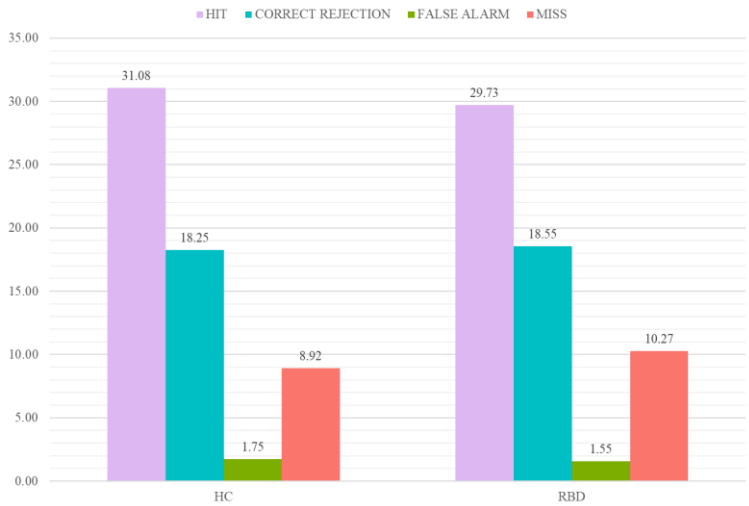


Figure 22. Graphical representation of the mean number of hits (pink), correct rejections (blue), false alarms (green) and misses (red) of HC (on the left) and iRBD patients (on the right) in the adaptive tapping task. Abbreviations: HC, healthy controls; iRBD, isolated rapid eye-movement sleep behavior disorder.

5.3.5. Resting-state EEG results

The correlation analysis between SMT behavioral data and resting-state EEG data showed a significant positive correlation between the frequency of the SMT pre-tasks and the location of the max peak on the resting-state EEG FFT (r = 0.968, p < 0.001) (see Figure 23).

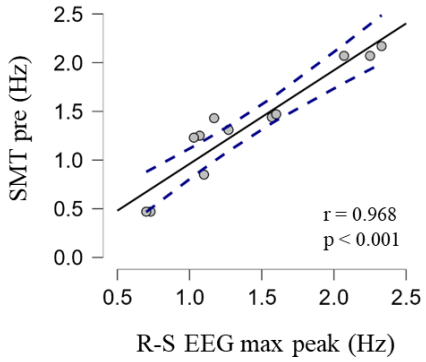


Figure 23. Scatter plot illustrating the significant positive correlation between the frequency of the SMT pre-tasks behavioral data (Hz) and max peak on the resting-state EEG data (Hz) selected on the basis of the individual's behavioral frequency. Abbreviations: EEG, electroencephalography; Hz, Heartz; R-S, resting-state; SMT, spontaneous motor tapping.

5.4. Discussion

The main aim of the present study was to investigate the possible presence of temporal processing and sensorimotor synchronization dysfunctions in a cohort of PSG-confirmed iRBD patients using a computerized version of three subtests of the BAASTA, namely the SMT task (i.e., time production), the anisochrony detection with tones task (i.e., time perception) and the adaptive tapping task (i.e., time production) (Dalla Bella et al., 2017). Based on previous literature (Brinkmann et al., 2021; Dalla Bella et al., 2017; Schwartze, Keller, et al., 2011; Schwartze, Rothermich, et al., 2011), the dimensions that define a "good timer" were identified, namely tapping rates (ITIs) as expected from the different tasks, low variability (low CV), short RTs, high detection performance and quick adaptation to tempo changes. We hypothesized that, while HC would show a certain degree of consistency between the respective measures that are indicative of optimal rhythm rates, iRBD patients would present impaired timing performances in line with previous findings (Cochen De Cock et al., 2020). Overall, both iRBD patients and HC showed good performances in all tasks, however with some interesting differences.

Concerning SMT tasks, no differences between HC and iRBD groups were observed on ITIs, SD and CV both pre and post tasks. Although not significant, HC showed a slower SMT pre tasks compared to iRBD patients, which is in line with previous studies both in iRBD (Cochen De Cock et al., 2020) and PD (Rose et al., 2020; Yahalom et al., 2004) patients and it has been hypothesized to be mainly related to gait festination, thus possibly representing an early progression biomarker in iRBD population. Moreover,

both HC and iRBD groups significantly differed when comparing SMT pre and SMT post tasks performances, showing a SMT post tasks ITI centered around 600 ms. This not only suggests that the SMT post tasks can no longer be defined as completely spontaneous, as it seemed to be influenced by previous perceived and produced rhythms, but also that there was a realignment of the SMT post tasks with the previously completed tasks (i.e., the anisochrony detection with tones and the adaptive tapping tasks) which presented rhythms centered around the optimal zone of 600 ms IOI (range 300-800 ms IOI) (Baruch et al., 2004; Fraisse, 1963; Schwartze et al., 2011). In line with these results, for both iRBD and HC groups we found that the PPT was around 600 ms (517 ms and 632 ms respectively); however, no significant correlations were observed between the PPT and SMT performances as instead demonstrated in previous studies (McAuley et al., 2006). Further corroborating these findings significant correlations were found between SMT post tasks and the performance on the adaptive tapping task; however, results highly differed between HC and iRBD groups. Indeed, HC showed significant positive correlations between the rate of the SMT post-tasks and the rate of both synchronization and continuation phases of the adaptive tapping task. On the contrary, the rate of the SMT post-tasks in the iRBD group was significantly inversely associated with the synchronization phase of the adaptive tapping task. We hypothesized that these results may suggest differences in adaptive motor control strategies between HC and iRBD patients. Indeed, in the adaptive tapping task the synchronization phase requires the alignment of the individual motor rhythm with an external rhythm, which then should be retain in the working memory not only in order to adapt to eventual time shifts but also to reproduce the same rhythm during the continuation phase (Schwartze et al., 2011). Thus, it can be speculated that HC effectively learned and internalized external rhythmic patterns during the adaptive tapping task, thus not only facilitating synchronization, adaptation and continuation of motor rhythms during the task but also influencing later SMT rhythm. This process most likely engages the PFC and related connections. As previously outlined, the PFC plays a key role in organizing and interpreting time-related information not only by maintaining and manipulating temporal sequences in working memory, but also by establishing a preparatory set through the prediction of the timing of future events (Fuster, 1995, 2001) (see Figure 17 for a complete overview of the underlying mechanisms). This executive-related process may be impaired in iRBD

patients; indeed, executive dysfunction is not only considered a hallmark of the disorder, but it is also an important marker of progression within the α-synucleinopathies spectrum (Leitner et al., 2024). Notably, in our recent meta-analysis (Leitner et al., 2024) we demonstrated that processing speed is the most severely impaired process within the executive function domain in iRBD patients. Notably, this finding is in line with previous studies in PD showing bradyphrenia and deficits in working memory in these patients (Johnson et al., 2016; Leitner et al., 2024; Shipley et al., 2002). In this context, it is worth mentioning the study published in 2021 by Arroyo and collaborators (Arroyo et al., 2021; Leitner et al., 2024) which reported a more severe executive dysfunction in PD patients involving the earliest stages of processing, specifically related to the motor and perceptual-alertness components. These findings suggest the role of an underlying executive dysfunction which may further contribute to timing-related impairments in iRBD patients.

Despite these interesting results, both synchronization and continuation phases performances were as expected from the adaptive tapping task for both groups. Moreover, no significant differences were found in the anisochrony detection with tones task when comparing the two groups. This evidence may suggest that iRBD patients do not present impairments in the detection of time shifts; although this is in contrast with previous findings (Cochen De Cock et al., 2020).

Finally, a significant positive association was found between the frequency of the SMT pre-tasks and the location of the max peak on the resting-state EEG. This is only a preliminary finding, but it may suggest the existence of a relationship between spontaneous rhythms during SMT and resting-state rhythms. This alignment might reflect an individual's intrinsic rhythmicity affecting both SMT and resting-state neural oscillatory activities. In line with this hypothesis, a pre-print study by Sun and colleagues (Sun et al., 2024) suggested that individual's intrinsic rhythmicity is also strictly linked to specific neural oscillatory patterns among which the individual resting alpha peak frequency (IAPF). IAPF is defined as the individual's most dominant frequency within the alpha band (i.e., 8 – 12 Hz) (Bazanova & Vernon, 2014; Mioni et al., 2020). Previous studies suggested that there is a strong association between alpha activity and individual's dominant rhythmicity (Bazanova, 2012) and that the neuromodulation of IAF may induce changes in time perception (Mioni et al., 2020). One question that naturally arises is

whether this individual rhythmicity may have a significant effect also on other rhythm-related tasks (such as the one included in the present study) and whether if it may differ between HC and iRBD. In this context, the identification of group-specific oscillatory timing profiles may provide important insights for the advancement and development of new rhythmic timing interventions. Indeed, previous studies on PD demonstrated the efficacy of rhythmic auditory stimulation not only on disturbances of gait, but also on perceptual and sensorimotor timing skills (Benoit et al., 2014; Dalla Bella et al., 2015), which may be beneficial also in the iRBD context and may contribute to the early identification of these patients.

The results of the present pilot study must be interpreted with caution, indeed there are some major limitations. First, the small sample size of both HC and iRBD groups, which limited the interpretability of the results. Second, it was not possible to match HC and iRBD for age, which significantly differed between the two groups. Despite all the between-groups analyses where adjusted for it, age difference may have had a strong impact on our results. Third, part of the aim of the present study was also to evaluate brain oscillations and EEG activities involved in the perception and reproduction of time intervals in both iRBD patients and HC in order to generate different timing profiles. Since data collection is still ongoing, only a preliminary analysis on resting-state EEG in HC was possible. However, HC EEG data were acquired not only during resting-state but also during task-performance. Moreover, high-density EEG and behavioral data collection for both age-matched HC and iRBD patients should start soon in order to extend the findings of the present pilot study.

In conclusion, the present pilot study suggested the presence of an executive-based dysfunction underlying timing-related impairments in iRBD patients. Moreover, it provided further insights into the nature of individual's intrinsic rhythmicity, which may represent an important target not only for future specific rhythmic timing interventions, but also for the early identification of disease biomarkers in the context of iRBD.

6. CONCLUSIONS

The main objective of my PhD dissertation was to explore the role of behavioral, neuropsychological and neurophysiological measures, as possible early markers for disease progression in iRBD patients.

The first chapter provided a comprehensive overview of the whole α-synucleinopathies spectrum: starting from its neurobiological bases and continuing by describing the main conditions that fall under this category. This chapter introduced the concept of iRBD as a prodromal stage of α-synucleinopathies, placed at the beginning of this continuum, and highlighted the importance of identifying not only risk factor and timely biomarkers of conversion, but also protective factors against neurodegeneration.

In the second chapter the specific aims of each study included in the present dissertation were described, and the main findings will be outlined in the following paragraphs.

The studies included in the third chapter aimed to set the cornerstone of this huge project by exploring the role of CR in iRBD in two ways. First, we evaluated the impact of CR on the cognitive functioning of these patients, demonstrating that iRBD patients with a high-level of CR showed the lowest percentage of MCI, and the best performance in visuo-constructive and verbal memory functions. Thus, we provided the first preliminary findings supporting CR as a possible protective factor in iRBD. Second, we combined CR and BR measures in order to evaluate their interaction and influence on pathological burden and cognitive performance in iRBD. We not only replicated previous evidence linking SW disruption to cognitive impairment, but we also suggested a novel interaction between pathological burden (i.e., PDRP) and SW sleep dynamics in iRBD. However, we did not find support for the potential role of CR in this context which could be attributed to several limitations, including the cross-sectional design of the study, the inherent issues of the CRIq, and the chosen proxy for measuring pathological burden (i.e., the PDRP). For these reasons, future longitudinal studies should test CR within a multimodal approach combining different measures for the evaluation of the pathological burden and CR.

The study included in the fourth chapter was the first to evaluate the PLR in iRBD patients compared to HC using a portable pupillometer. The results demonstrated the role

of pupillometry as a potential supportive tool in the detection of early peripheral cholinergic dysfunction associated with cognitive impairment in iRBD. Specifically, the presence of PLR alterations was associated with poorer performances in visuospatial and visuoperceptive processing, further supporting the presence of an underlying cholinergic dysfunction that may be associated with a more malignant conversion phenotype. Despite these interesting results, the study presented several limitations, among which the cross-sectional design of the study, the fact that HC and iRBD were not matched for age, the presence of possible confounding factors (e.g., caffeine/nicotine consumption, time of the day, sleepiness) and the absence of the ACmax index. Given these premises, future studies should investigate the PLR within the entire α-synucleinopathies spectrum in a longitudinal study design and considering all the above-mentioned confounding in order to test the evolution of the PLR from the prodromal to the final stages of disease within an ideal experimental setting.

Finally, the fifth chapter included a pilot study with the aim of evaluating whether temporal processing and sensorimotor synchronization dysfunctions may be already present in iRBD patients and to investigate resting-state EEG-related rhythms. The results suggested the presence of an executive-based dysfunction underlying timing-related impairments in iRBD patients. Moreover, it provided further insights into the nature of individual's intrinsic rhythmicity, which may be an important target not only for future specific rhythmic timing interventions, but also for the early identification of disease biomarkers in the context of iRBD. Given the pilot nature of the study, it presented some major limitations among which the cross-sectional design of the study, the small sample size, the impossibility to match HC and iRBD for age and the limited EEG analysis on HC.

Altogether these different aspects of iRBD pathology shape a complex puzzle of the different dysfunctions, pathophysiological causes, disease mechanisms and future clinical trajectories that these patients may come across in their path. As previously mentioned, for patients and their loved ones, living with iRBD may be compared to living with a Damocles sword hanging over their heads: the disease exhibits an extremely high conversion rate, yet the precise timing, form, or certainty of its eventual phenoconversion remains indeterminate.

In the end…

"*All we have to decide is what to do with the time that is given us"*

(Tolkien, 1954)

…and the same goes for our patients. The present PhD dissertation adds a little piece to the puzzle of the current knowledge in this field. In this regard, it should be acknowledged that although the studies included in this dissertation utilized practical and easily accessible validated tools, which are optimal for standardizing research, they often fail to fully capture the complexity and inter-subject variability experienced in everyday clinical practice. Nevertheless, I hope that my thesis will contribute to paving the way for future therapeutic research.

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