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Prevalence of idiopathic Rapid Eye Movement Sleep Behavior Disorder (RBD) and possible novel retinal biomarkers of alpha-synucleinopathy.

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1. Introduction

Rapid Eye Movement (REM) Sleep Behavior Disorder (RBD) is a parasomnia, characterized by the presence of abnormal behaviors during the REM sleep phase. It has been formally described for the first time in 1986 in a group of five patients that experienced complex movements during REM sleep phase, including punching, kicking and violent stereotypies associated with a vivid dream content, often reporting aggressive experiences [1]. In this original report, patients were previously diagnosed with either psychiatric disorders, epilepsy or sleep apnoeas, and only the observation of these behaviors in a controlled environment using a polysomnography (PSG) allowed the classification of these movements to arise from the REM sleep phase, with a pathological concurrent increase in the muscle activity over the chin muscle during the REM sleep [1]. Ten years later, the same group described a higher risk for RBD patients to develop Parkinson's Disease (PD), thus suggesting that RBD could be considered a prodromal phase of PD [2].

1.2 Epidemiology

RBD can be classified as isolated (former "idiopathic") RBD (iRBD) or secondary RBD, when associated to a preexisting condition such as PD, Multiple System Atrophy (MSA), Lewy Body Dementia (LBD) or narcolepsy.

iRBD has a low prevalence in the general population, with estimates obtained using PSG ranging from 0.3% to 1.15% [3,4]. Prevalence rates are higher when

diagnosing iRBD on the basis of either a questionnaire or a clinical examination, ranging from 4.6% to 13.6% [5,6] with a concordance across different studies regarding a male prevalence of the disease [7], which is significantly stronger in some studies [4,8] and milder in others, indicating that there could be some factors responsible for the difference seen in sex prevalence [9,10]. One of the most frequent explanation is the referral bias, due to the fact that men tend to exhibit more violent behaviors during sleep and thus seek medical advice more frequently than women [10].

iRBD risk has been associated with a lower level of education and a history of head injury [8,11,12]. Among environmental risk factors exposure to pesticides [11] and working in mines [8] seems to increase the risk of being diagnosed with iRBD. Several of these risk factors are also shared by PD patients, but when investigating other known risk factors for PD such as caffeine intake, no association was found [11], and even an inverse association with smoking has been described, increasing the risk of iRBD rather than being a protective factor [11,12]. While there is no description of a genetic mutation associated with iRBD, an increased frequency of GBA mutation has been described in iRBD subjects [13], with evidences towards the presence of a genetic component that might modulate time to phenoconversion [14].

1.3 Pathophysiology

The first experiments that delved into the physiopathology of RBD have been conducted in cats that underwent a pontine lesion and exhibited a normal REM sleep architecture with the absence of the expected muscle atonia and the presence of complex behaviors [15]. In the following years, a large body of evidence has implicated other structures in the physiopathology of REM sleep and RBD, describing a complex network involving both brainstem and cortical regions (Figure 1). In healthy individuals, REM sleep is initiated by the combined activation of posterior lateral hypothalamus (PH), the dorsal paragigantocellular reticular nucleus (DPGi) and the ventrolateral periaqueductal grey (VLPAG), which inactivate several wake-promoting nuclei[16]. VLPAG also activates the sublaterodorsal tegmental nucleus (SLD) that projects excitatory stimuli towards the ventral medulla nuclei (raphe magnus nucleus, RMg; ventral gigantocellular, GiV and lateral paragigantocellular, LPGi) which in turn inhibit the spinal motoneurons via GABAergic and glycinergic synapses [16–18] generating the physiologic atonia during REM sleep. In RBD, the degeneration of the SLD causes the lack of inhibition of spinal motoneurons, which receive excitatory impulses coming from the motor cortex leading to REM Sleep Without Atonia (RSWA) and abnormal movements during sleep [19].

The range of movements exhibited by RBD patients spans from simple twitching to complex behaviors, associated with oneiric activity, thus implicating the role

of higher (cortical) functions. Indeed, during REM sleep limbic cortical structures are active, such as the medial entorhinal cortex (mENT), the anterior cingulate cortex (ACA), the dentate gyrus (DG) and retrosplenial cortex (RSC). The latter has a fundamental role in dream production, and might promote complex motor behaviors by activating the motor cortex whose excitatory impulses are then transmitted to the spinal motoneruons and acted out due to the lack of inhibition [18].
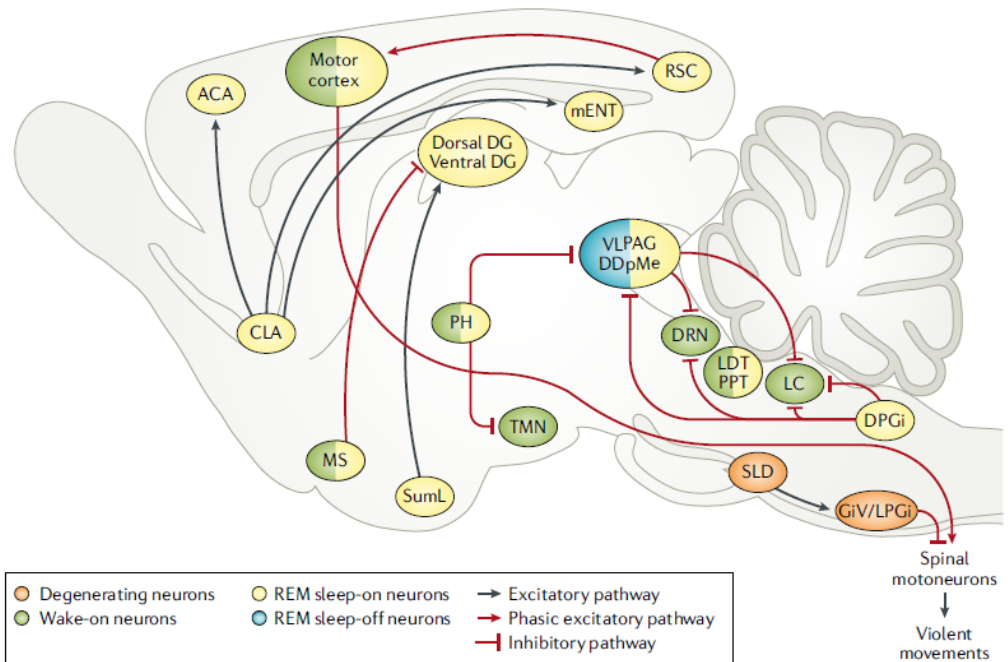

Figure 1. Neuronal network generating REM sleep and inducing REM sleep without atonia and RBD. Legend: Posterior lateral hypothalamus (PH); dorsal paragigantocellular reticular nucleus (DPGi); ventrolateral periaqueductal grey (VLPAG); Tuberomammillary nucleus (TMN); locus coeruleus (LC); dorsal raphe nucleus (DRN); dorsal deep mesencephalic nuclei (DDpMe); sublaterodorsal tegmental nucleus (SLD); raphe magnus (RMg); ventral gigantocellular nucleus (GiV); alpha gigantocellular
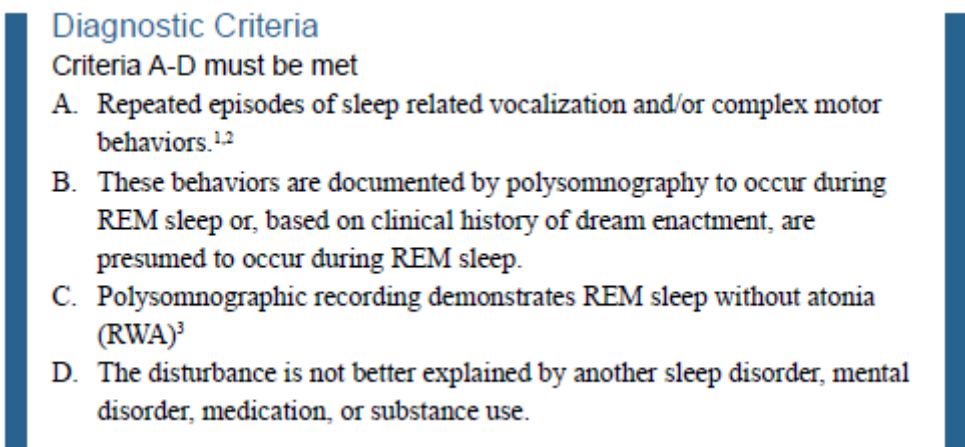
nucleus (GiA); lateral paragigantocellular nucleus (LPGi); retrosplenial cortex (RSC); medial entorhinal cortex (mENT); anterior cingulate cortex (ACA); dentate gyrus (DG); claustrum (CLA); lateral supramammillary nucleus (SumL); medial septum (MS); laterodorsal tegmental nucleus (LDT); pedunculopontine tegmental nucleus (PPT) – adapted from *Dauvilliers et al 2018*

1.4 Clinical characteristics

The main clinical feature of RBD patients is represented by the presence of repetitive movements, ranging from simple to complex, during REM sleep, often associated with the recall of an unpleasant dream experience (e.g. being attacked, running away from a danger, etc.), termed Dream Enacting Behaviors (DEBs). Dream content of RBD patients has features of vividness, danger and defence from harm, in higher frequency when compared to the general population [20]. However, recent studies have challenged this hypothesis, demonstrating that the dream content of RBD patients (either idiopathic or in PD associated RBD) does not differ from the general population [21] or from PD patients without RBD [22]. DEBs encompass a large variety of movements, from simple kicking and punching, to complex vocalizations (such as singing) or behaviors (e.g. attacking a threat with an imaginary knife). DEBs may result in injury to the patient or to the bed-partner, depending on the intensity and the complexity of the movement. In severe cases, patients might report fractures or head trauma with subdural hematomas after falling from the bed during an episode.

1.5 Diagnosis

Diagnosis of RBD is made according to the International Classification of Sleep Disorders – Third Edition and requires the execution of a videopolysomnography (VPSG) to identify the presence of RSWA and REM associated abnormal behaviors [23] (Figure 2).

Diagnostic Criteria

Criteria A-D must be met

A. Repeated episodes of sleep related vocalization and/or complex motor behaviors.[1,2]
B. These behaviors are documented by polysomnography to occur during REM sleep or, based on clinical history of dream enactment, are presumed to occur during REM sleep.
C. Polysomnographic recording demonstrates REM sleep without atonia (RWA)[3]
D. The disturbance is not better explained by another sleep disorder, mental disorder, medication, or substance use.

Figure 2. Diagnostic criteria for RBD - ICSD 3

Presence of RSWA has been usually assessed visually through the analysis of the phasic or tonic activity of the mentalis muscle. An alternative approach studying also limb muscles, showed a higher sensibility in demonstrating an increased muscle phasic activity during REM sleep in RBD patients [24]. Moreover, the ICSD-3 introduced the definition of "provisional RBD", to be used to make a provisional diagnosis of RBD in patients who exhibit a history of DEBs and the presence of typical movements during REM sleep but fail to reach the cut-off scores for the presence of RSWA.

When VPSG is not available, or patients do not want to undergo the exam, a diagnosis of probable RBD (pRBD) can be sought. pRBD is usually diagnosed either with an extensive clinical examination or using validated questionnaires such as the REM Sleep-Behavior Single-Question Screen (RBD1Q) [25] , the RBD Screening questionnaire (RBDSQ) [26] or the Mayo Sleep Questionnaire [27]. However, questionnaire might diagnose as RBD patients suffering from RBD mimics, such as Obstructive Sleep Apnoeas (OSA) that might present RBD-like movements, or patients with other NREM parasomnias.

1.6 Therapy
The main objectives of RBD treatment are to reduce the risk of movement related injuries, and to improve the quality of life of the bed partner. Non pharmacological interventions should be suggested to patients, such as removing dangerous objects from bedside, or using bed rails to reduce the risk of falling from the bed during DEBs [28]. Pharmacotherapy is usually started when patients and/or bedpartners report sleep disruption. The drug of choice is clonazepam, which has been shown to reduce the frequency and intensity of DEBs when regularly taken before sleeping [28,29]. However, the wide range of clonazepam side effects, including cognitive dysfunction, sleepiness and worsening of preexisting OSA, suggests a careful use in patients with an advanced age. Another treatment option with less side effects and a similarly good profile of

responsiveness is represented by melatonin, an over the counter medication, that has demonstrated an effect on RBD comparable to clonazepam [18,28].

1.7 RBD and alpha-synucleinopathies

In the last 20 years increasing evidences from epidemiological studies have shown that iRBD patients have a higher risk of developing a neurodegenerative disease, especially alpha-synucleinopathies such as PD, DLB and MSA [30]. In a recent multicenter study, it has been shown that almost 28% of iRBD patients develop a neurodegenerative disease with a mean latency of 4.6 years from RBD diagnosis [7], converting equally to PD or DLB. Studies conducted in longitudinal cohorts with longer follow-up demonstrated that up to 90% of patients develop a neurodegenerative disease almost 14 years after RBD diagnosis [31]. This has led to the definition of iRBD as the most specific risk factor for the development of PD and it represents the strongest prodromal marker in the diagnosis of "Prodromal PD" [32]. Indeed, in the context of an established PD, prevalence of RBD has been estimated to be 42.3% [33] and has been associated with a worse cognitive performance [34], an akinetic-rigid phenotype [35], with the presence of orthostatic hypotension [36] and an overall increased non-motor symptoms burden [37], configuring a distinct PD phenotype [38].

Patients with iRBD frequently exhibit non-motor symptoms (NMS), typical of PD, such as personality disorders, hyposmia, dysautonomia, constipation and

cognitive impairment, suggesting that the two disease entities might share a common underlying pathology [32,39]. Moreover, iRBD patients displaying several of these NMS such as hyposmia, cognitive impairment, constipation and colour vision disturbances have a higher risk of phenconversion to an alpha-synucleinopathy [7].

Considering that the median time of phenoconversion from iRBD to an alpha-synucleinopathy has been estimated to be eight years [7], and that this interval might represent a relevant window of time for testing the efficacy of neuroprotective drugs [40], a large effort has been devoted towards the identification of the most sensible biomarker of future conversion to an alpha-synucleinopathy in iRBD, either clinical or instrumental [41].

1.8 List of PhD projects
During the three years of my PhD project, I have worked on elucidating the prevalence of idiopathic REM sleep behavior disorder and finding a novel biomarker of alpha-synucleinopathy. The three main objectives are:

- To describe the global prevalence of iRBD through a systematic review and meta-analysis of published studies (Article 1);

- To provide the estimate of the prevalence of iRBD in the city of Catania (Article 2)

- To assess the use of retinal layer thickness as a biomarker of alpha-synucleinopathy in iRBD (Article 3)

2.0 Article 1

Prevalence of idiopathic REM behavior disorder: a systematic review and meta-analysis.

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Abstract

Study Objectives

To provide an overall estimate of the prevalence of idiopathic REM Sleep Behavior Disorder (iRBD).

Methods

Two investigators have independently searched the PubMed and Scopus databases for population-based studies assessing the prevalence of iRBD. Data about type of diagnosis (polysomnographic diagnosis, defined iRBD [dRBD]; clinical diagnosis, probable RBD [pRBD]), continent, age range of the screened population, quality of the studies, sample size, screening questionnaires and strategies have been gathered. A random effect model was used to estimate the pooled prevalence. Heterogeneity was investigated with subgroup analysis and meta-regression.

Results

From 857 articles found in the databases, 19 articles were selected for the systematic review and meta-analysis. According to the type of diagnosis, five studies identified dRBD cases given a pooled prevalence of 0.68% (95%CI 0.38-1.05) without significant heterogeneity (Cochran's Q p=0.11; $I^2 = 46.43\%$). Fourteen studies assessed the prevalence of pRBD with a pooled estimate of

5.65% (95%CI 4.29-7.18) and a significant heterogeneity among the studies (Cochran's Q p<0.001; I^2 = 98.21%). At the subgroup analysis, significant differences in terms of prevalence were present according to the quality of the studies and, after removing two outlaying studies, according to the continents and the screening questionnaire used. Meta-regression did not identify any significant effect of the covariates on the pooled estimates.

Conclusion

Prevalence estimates of iRBD are significantly impacted by diagnostic level of certainty. Variations in pRBD prevalence are due to methodological differences in study design and screening questionnaires employed.

Introduction

Rapid eye movement sleep behavior disorder (RBD) is a sleep condition characterized by the presence of abnormal movements during REM sleep, frequently enacting dream content [1]. Data about prevalence of the disease are scarce, with few population-based studies often reporting estimates that differ significantly depending on the diagnostic process employed [2,3]. In fact, studies on the prevalence of idiopathic RBD (iRBD) conducted in the general population are often limited by the difficulties of the diagnostic process which requires, according to the American Academy of Sleep Medicine (AASM) criteria, a polysomnography (PSG) to detect the presence of decreased muscle atonia and sudden movements in sleep, that are a hallmark of the disease [4]. To overcome this limitation, several questionnaires have been developed to investigate the presence of iRBD for screening purposes with an acceptable sensitivity and specificity [5-7]. RBD subjects diagnosed using these instruments without a PSG confirmation are defined as probable RBD (pRBD). However, the screening questionnaires used have the tendency to include several false positive cases, biasing the estimates of iRBD prevalence. A study showed that questionnaires originally developed to screen for the presence of RBD in a hospital setting have a very low positive predictive value when applied on the general population [8].

In this context, we conducted a systematic review and meta-analysis of all the epidemiological studies on iRBD to summarize the existing evidence, provide an overall estimate of the prevalence of PSG defined RBD (dRBD) and pRBD, and exploring possible sources of heterogeneity.

Methods

Literature search

A comprehensive literature research has been conducted on PubMed and Scopus research databases by using the following combinations of keywords and Boolean operators: for PubMed: ("Rem behavior disorder" OR "Rem behaviour disorder" OR "Rapid Eye movement sleep behavior disorder" OR "Rapid eye movement sleep behaviour disorder" OR "RBD") AND ("Prevalence" OR "Incidence" OR "Epidemiology"); for Scopus: TITLE-ABS-KEY("Rem behavior disorder" OR "Rem behaviour disorder" OR "Rapid Eye movement sleep behavior disorder" OR "Rapid eye movement sleep behaviour disorder" OR "RBD") AND TITLE-ABS-KEY("Prevalence" OR "Incidence" OR "Epidemiology"). No filter was applied on the publication date of the articles and all the results up to April 2020 were included. We considered articles published in English, French, Spanish or Italian language.

Inclusion criteria were defined as:

1. Population-based studies;

2. Studies in which the methods for diagnosing iRBD are clearly stated;

3. Studies with extensive description of the recruitment process.

Studies assessing the presence of secondary RBD or studying the prevalence of RBD in patients with a previous diagnosis of a neurodegenerative disorder have been excluded.

Systematic reviews and meta-analyses were examined for additional references. Two neurologists (CEC, LG) independently conducted the literature search. After duplicate removal, titles, abstracts and full texts were screened independently. Disagreements over inclusion of selected articles were discussed and supervised by a third neurologist (AN). Corresponding authors were contacted for additional data when necessary.

Data extraction

From the included articles, we collected information about country, age range, study design, sampling method used, number of diagnostic stages for iRBD diagnosis, screening instruments used, diagnostic certainty of iRBD diagnosis (dRBD or pRBD) and prevalence data. We calculated the prevalence of iRBD

directly from the raw data in the text and/or tables in the original studies that did not provide this information.

Quality assessment

Quality of the prevalence studies has been rated according to established criteria [9]. Specifically, we used a scale composed by three items and eight subitems evaluating the representativeness of the sample (sample definition, ascertainment method, response rate, description of non-responders and the presence of any difference between the sample and the general population), the methods of assessment of the neurological condition (standardized assessment, use of validated instruments) and the statistical analysis (calculation of confidence intervals). Hypothesizing that quality of the studies might impact the prevalence estimates, studies were divided into high quality (5-8) and low quality (0-4).

Statistical analysis

The systematic review and meta-analysis followed the PRISMA and MOOSE guidelines (Tables S1 and S2) [10,11]. Prevalence was calculated as a percentage (number of cases by the surveyed population) along with their 95% confidence intervals (CI). We used Freeman-Tukey arcsin transformation for synthesizing

proportions [12]. Random-effect model was conducted according to the presence of significant heterogeneity, which was assessed through Cochran's Q-test ($p<0.1$) and I^2 statistics (greater than 25%) [13]. A stratified analysis was performed for dRBD and pRBD. Forest plots and pooled estimates were displayed.

Subgroup analyses were conducted for the following variables: continent, age range of the included population, number of diagnostic stages, type of questionnaire, sample size and quality of the studies. Meta-regression was also performed to investigate potential sources of heterogeneity considering several study-level covariates. Publication bias was evaluated through the Egger's test. Potential outliers and influential cases were examined [14]. Sensitivity analyses were performed after removing selected articles or outliers to explore the robustness of the findings. Two-sided p values <0.05 were considered statistically significant. Analyses were conducted using the computing environment R (version 3.6.2, package: metafor).

Results

Study selection

The literature research retrieved a total of 857 articles. After removal of duplicates and exclusion by title and abstract review, 19 articles have been selected for full text assessment. Four of them did not satisfy the inclusion criteria: one addressed subjects already complaining for sleep disorders [15], two were not applied on the general population [16,17], and another one reported the follow-up data of a prospective study [18] and thus we have included the original study [19]. Other three studies have been added from the references of the selected articles. Nineteen studies have been included for the qualitative and quantitative analysis (Figure 1).

Figure 1. Flowchart of the literature search

PRISMA 2009 Flow Diagram

Identification

Records identified through database searching
Pubmed n = 471
Scopus n = 386
TOTAL n = 857

Screening

Records after duplicates (n = 254) removed
(n = 603)

Records excluded after examining titles and abstracts
(n = 584)

Eligibility

Full-text articles assessed for eligibility
(n = 19)

Studies retrieved by references of other studies
(n = 4)

Full-text articles excluded, with reasons
(n = 4)

Included

Studies included in qualitative synthesis
(n = 19)

Studies included in quantitative synthesis
(meta-analysis)
(n = 19)

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

Study characteristics

The characteristics of the included studies are reported in Table 1. Considering the level of diagnostic certainty, five studies assessed the prevalence of dRBD [2,8,20-22] while 14 used the pRBD definition [3,19,23-34]. Ten studies have been carried out in Asia [2,20,22,23,25-27,32-34], four in North America [24,28-30] and five in Europe [3,8,19,21,31].

Table 1. Characteristics of the included studies.

First Author	Year	Country	RBD diagnostic level	Age of screened sample (years)	Stages	Screening questionnaire for RBD	Number of participants	Cases of iRBD	Prevalence	95% CI	Quality score
Chiu	2000	China	definite	>70	3	Sleep related injury single question	1034	3	0.29	0.06-0.85	6
Uemura	2011	Japan	probable	>60	1	RBDSQ	968	59	6.10	4.67-7.79	7
Boot	2012	USA	probable	70-89	1	Mayo Sleep Questionnaire	727	53	7.29	5.51-9.43	7
Kang	2013	South Korea	definite	>60	1	Not specified	348	4	1.15	0.31-2.92	6
Noyce	2013	UK	probable	60-80	1	RBDSQ	1324	43	3.25	2.36-4.35	3
Mahlknecht	2015	Italy	probable	>60	2	RBDSQ and RBD - Innsbruck	456	21	4.61	2.87-6.95	8
Nomura	2015	Japan	probable	>20	2	RBDSQ	1572	9	0.57	0.26-1.08	6
Ma	2016	China	probable	>20	1	RBD1Q	19614	1411	7.19	6.84-7.56	5
Wong	2016	China	probable	>18	1	RBD HK	12784	724	5.66	5.27-6.08	4
Ma	2017	China	probable	>50	1	RBDSQ	3635	98	2.70	2.19-3.28	7
Pujol	2017	Spain	definite	>60	3	RBD1Q	539	4	0.74	0.20-1.89	7
Haba-Rubio	2018	Switzerland	definite	35-75	2	MUPS	1997	21	1.05	0.65-1.60	8
Yao	2018	Canada	probable	45-85	1	RBD1Q	19584	958	4.89	4.59-5.20	8
Shprecher	2019	USA	probable	>50	1	RBD1Q	1406	191	13.58	11.84-15.49	2
Aye	2020	Singapore	probable	>50	1	Self-reported questionnaire from MDS criteria for prodromal PD	392	24	6.12	3.96-8.97	1
Baldin	2020	Italy	probable	>60	1	Mayo Sleep Questionnaire	392	17	4.34	2.55-6.85	5
Hattori	2020	Japan	probable	> 18	1	RBDSQ	4953	596	12.03	11.14-12.97	4
Sasai-Sakuma	2020	Japan	definite	>65	3	RBD1Q	1464	8	0.55	0.24-1.07	6
Shprecher	2020	USA	probable	>0	1	RBD1Q	439	31	7.06	4.85-9.87	4

Legend: UK: United Kingdom; USA: United States of America; RBDSQ: RBD screening questionnaire; RBD1Q: RBD single question screen; RBD HK: RBD questionnaire Hong Kong; MUPS: Munich Parasomnia Scale; MDS UPDRS: Movement Disorders Society Unified Parkinson's Disease Rating Scale; PD: Parkinson's Disease.

Characteristics of dRBD studies

Considering dRBD studies, three out of five studies recruited cases from a population sample size >1000 [20-22]. All of the studies screened subjects older than 60 years except for the study by Haba-Rubio et al. [21] whose age range was from 35 to 75 years. Two studies had a participation proportion higher than 70% [8,21], and one had a value close to it (68.8%) [20].

Out of the five studies that confirmed RBD through a videoPSG (VPSG), one study directly performed a VPSG to the entire selected population [2]. In another study, part of a large population-based study on sleep disorders [35], the entirety of the recruited population underwent a PSG examination [21]; suspected RBD cases were found through the use of the answers to the RBD related items of the Munich Parasomnia Screening (MUPS) questionnaire [36] and the final diagnosis of dRBD was obtained after the analysis of their PSG recordings. A three-stage design was used in the other three studies: screening questionnaire followed by clinical examination and VPSG analysis on highly suspected cases [8,20,22]. Of these, one used a questionnaire aimed at evaluating the presence of sleep related injuries [20] while the other two performed the screening with the RBD One question (RBD1Q) [8,22].

VPSG evaluation of RBD was based on SINBAR criteria for three studies [8,21,22] while the other two used the ICSD-2 criteria [2] and the Mahowald 1994 criteria [37,20].

Overall, the dRBD studies were of high quality, ranging from 6 [2,20,22] to 7-8 [8,21].

Characteristics of pRBD studies

Among studies on the prevalence of pRBD, only four out of 14 had as primary or secondary aim the evaluation of pRBD prevalence [3,25,27,30]. Two evaluated risk factors associated with pRBD [26,28], one considered pRBD as a risk factor for the development of Mild Cognitive Impairment (MCI) and Parkinson's Disease (PD) [24] and one gave the estimates of pRBD prevalence while validating the Japanese version of the RBD Screening Questionnaire (RBDSQ) [34]. The remaining six studies considered pRBD among other Mild Parkinsonian Signs (MPS) [19,23,29,31-33].

Sample sizes of the studies varied widely, with the majority of them screening a population larger than 1000 subjects [19,25-29,33,34]. Four studies included a screened population of less than 40 years of age [26,30,33,34], while the other ten

included only subjects older than 40 years. Participation rate was higher than 70% for half (n=7) studies [3,19,23,26,28,31,32].

The majority of pRBD studies (n=12) used a one-stage approach through the use of different validated questionnaires [19,23-33], except for two using a two-stage approach [3,34]. In particular, as second stage, one used a full clinical examination by a movement disorder and a sleep specialist [3] while the other a telephone interview by a neurologist [34]. The most frequently used screening questionnaire was the RBDSQ (n=6) (Table 1). Only one study used a non-validated screening questionnaire for RBD adapted from the Movement Disorders Society Unified Parkinson's Disease Rating Scale (MDS UPDRS) scale [32].

According to the quality assessment, eight studies had a high quality (5-8) [3,23-25,27,28,31,34], and six a low quality (≤4) [19,26,29,30,32,33].

Prevalence of dRBD

The overall prevalence of dRBD was 0.68% (95%CI 0.38-1.05) without significant heterogeneity among the studies (Cochran's Q p=0.11; I^2=46.43%) (Figure 2). We have conducted a sensitivity analysis after excluding the study conducted by Chiu et al. [20] that defined iRBD after searching for sleep related injuries (SRI), finding

a close prevalence 0.81% (95%CI 0.54-1.12) and a not significant heterogeneity

(Cochran's Q p=0.36; I^2=5.98%).

Figure 2. Forest plot for iRBD prevalence
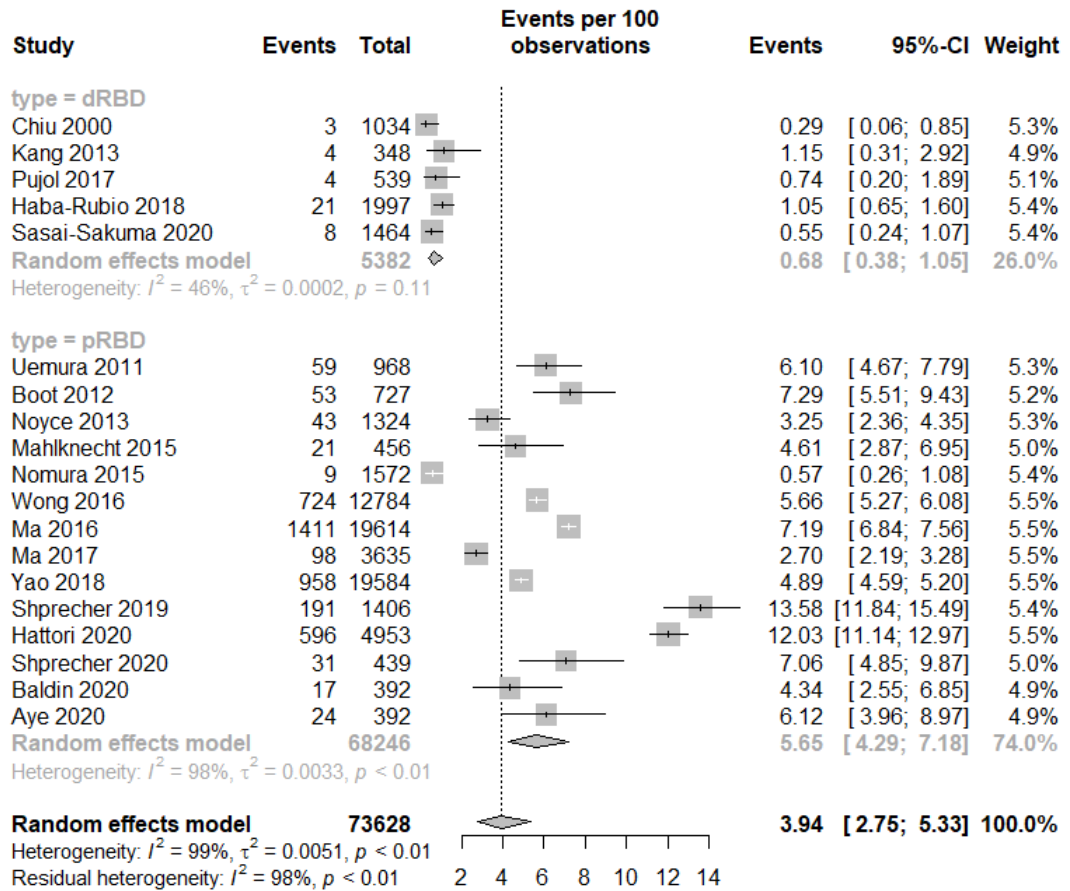

Legend. dRBD: defined RBD; pRBD: probable RBD.

Prevalence of pRBD

Prevalence of pRBD was 5.65% (95%CI 4.29-7.18) with a significant heterogeneity among the studies (Cochran's Q p<0.001; $I^2 = 98.21\%$). Results are displayed in Figure 2. In order to explore the causes of the high level of heterogeneity for pRBD studies, we performed several subgroup analyses.

Considering the continent, prevalence of pRBD was the lowest [3.75% (95% CI 2.89-4.72); $I^2 = 16\%$] for Europe (n=3), while Asia (n=7) and North America (n=4) displayed respectively 5.19% (95%CI 3.19-7.63; $I^2 = 99\%$) and 7.94% (95%CI 4.04-12.98; $I^2 = 98\%$), with a borderline significant difference at the subgroup analysis (p=0.081).

According to quality of the studies, prevalence was 7.60% (95%CI 4.62-11.25; $I^2 = 98\%$) for low quality studies (n=6) and 4.35% (95%CI 2.91-6.06; $I^2 = 98\%$) for high quality studies (n=8) with a borderline significant difference between the groups (p=0.066) (Figure 3).

**Figure 3.** Prevalence of pRBD according to quality of the studies.
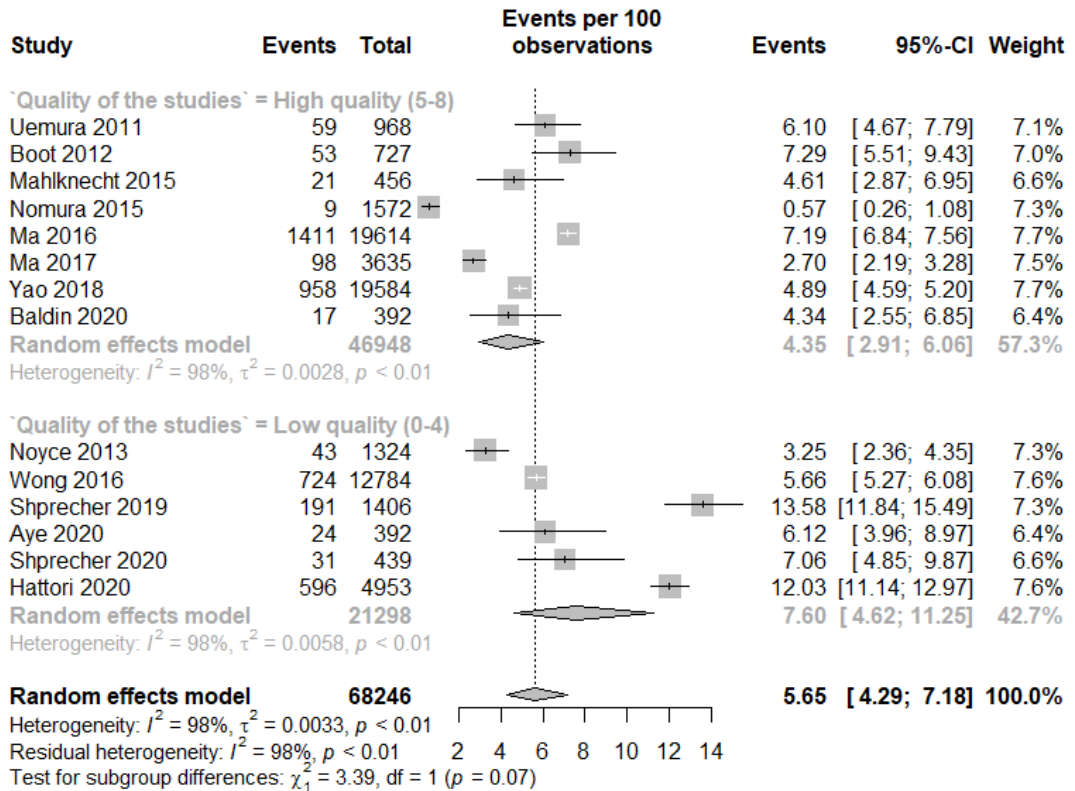
Studies were then classified according to the age range of screened subjects. Pooled estimates were 5.42% (95%CI 1.83-10.75; I^2 = 99%) for studies screening a population younger than 40 years (n=4), while for studies screening an older than 40 years population (n=10) prevalence was 5.74% (95%CI 4.35-7.31; I^2 = 97%). No significant difference was found by subgroup analysis (p=0.892).

According to the stages used for the diagnostic process, prevalence for the one-stage group (n=12) was 6.40% (95%CI 5.01-7.94; I^2 = 98%), while for the two-

stage group (n=2) prevalence was 2.1% (95%CI 0.00-7.8; $I^2 = 98\%$) without a significant difference (p=0.127) (Figure 4).

Figure 4. pRBD studies according to number of stages performed.
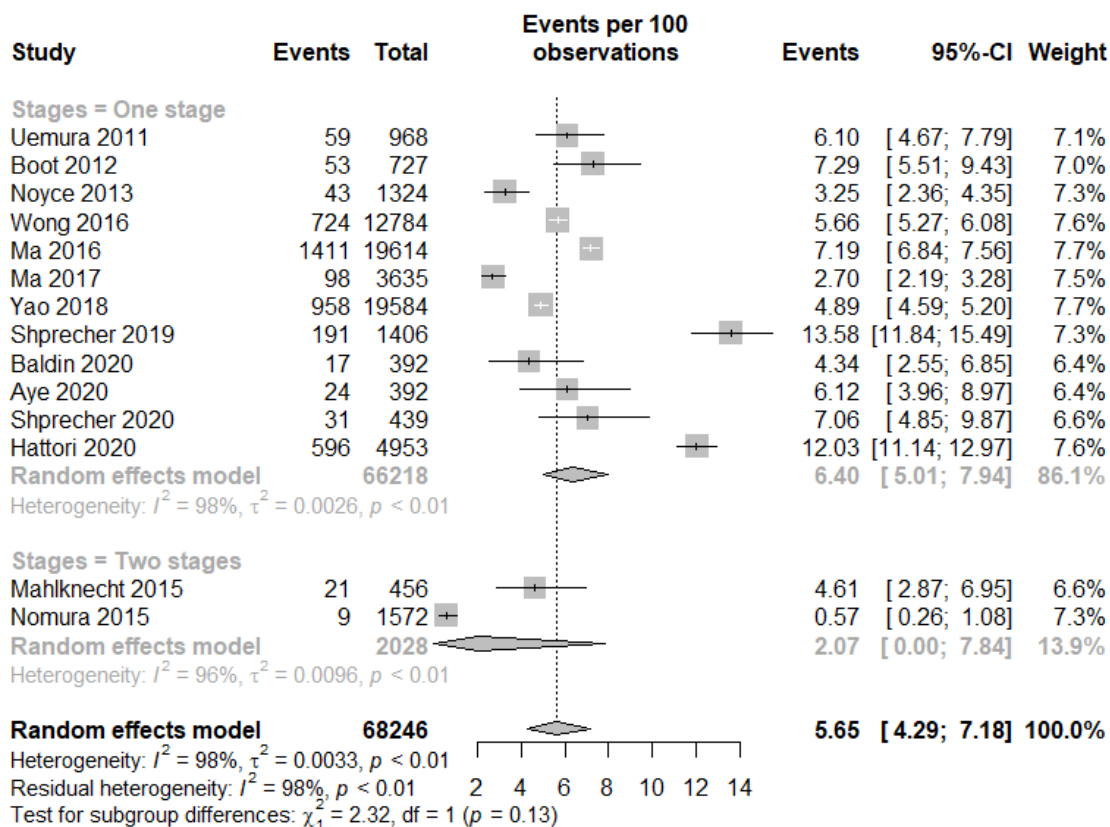
Prevalence of pRBD using different screening questionnaires was 7.86% (95%CI 5.56-10.53; $I^2 = 98\%$) for studies using RBD1Q (n=4); 4.23% (95%CI 1.26-8.82; $I^2 = 99\%$) for RBDSQ (n=6) and 5.82% (95%CI 4.94-6.76, $I^2 = 37\%$) for other questionnaires. Subgroup analysis did not find any significant difference (p=0.202).

For studies with a sample size of less than 1000 subjects (n=3), the estimated prevalence was 4.97% (95%CI 3.82-6.27; $I^2 = 0\%$), while for those with a larger sample size (>1000 subjects; n=11) prevalence was 5.81% (95%CI 4.27-7.57, $I^2 = 99\%$) without a significant difference across the subgroups (p=0.465).

No publication bias was identified (Egger's test p=0.817).

At the Baujat plot [14], two studies were identified as the most relevant contributors to the overall pRBD prevalence estimates heterogeneity [33,34]. As a sensitivity analysis, these two studies have been excluded and the pRBD prevalence pooled estimate was 5.82% (95%CI 4.72-7.03; $I^2 = 96\%$). Results of the subgroup analyses conducted without the two outlaying studies, were comparable to the general analysis, except that now a significant between group heterogeneity was found for the continents (p=0.05) and the questionnaires used (p=0.03) (Figures 5 and 6).

Figure 5. Sensitivity analysis, pRBD studies classified according to the

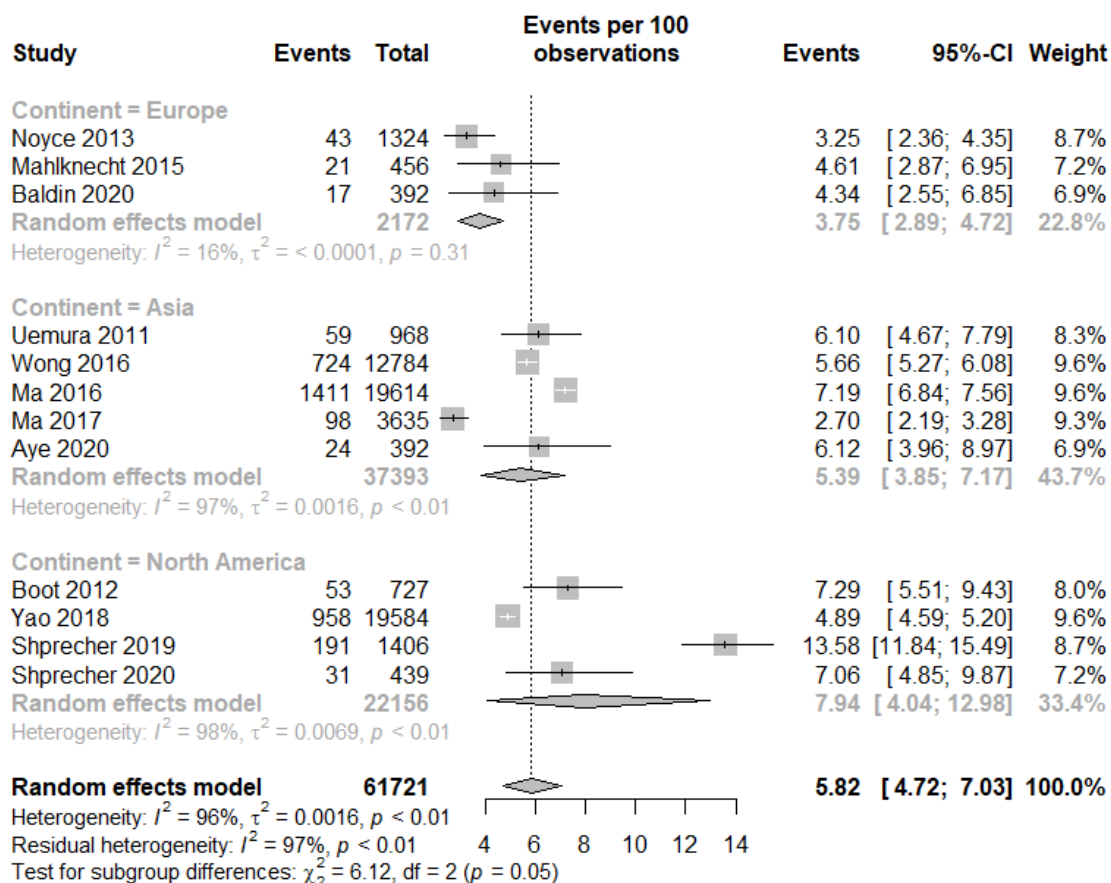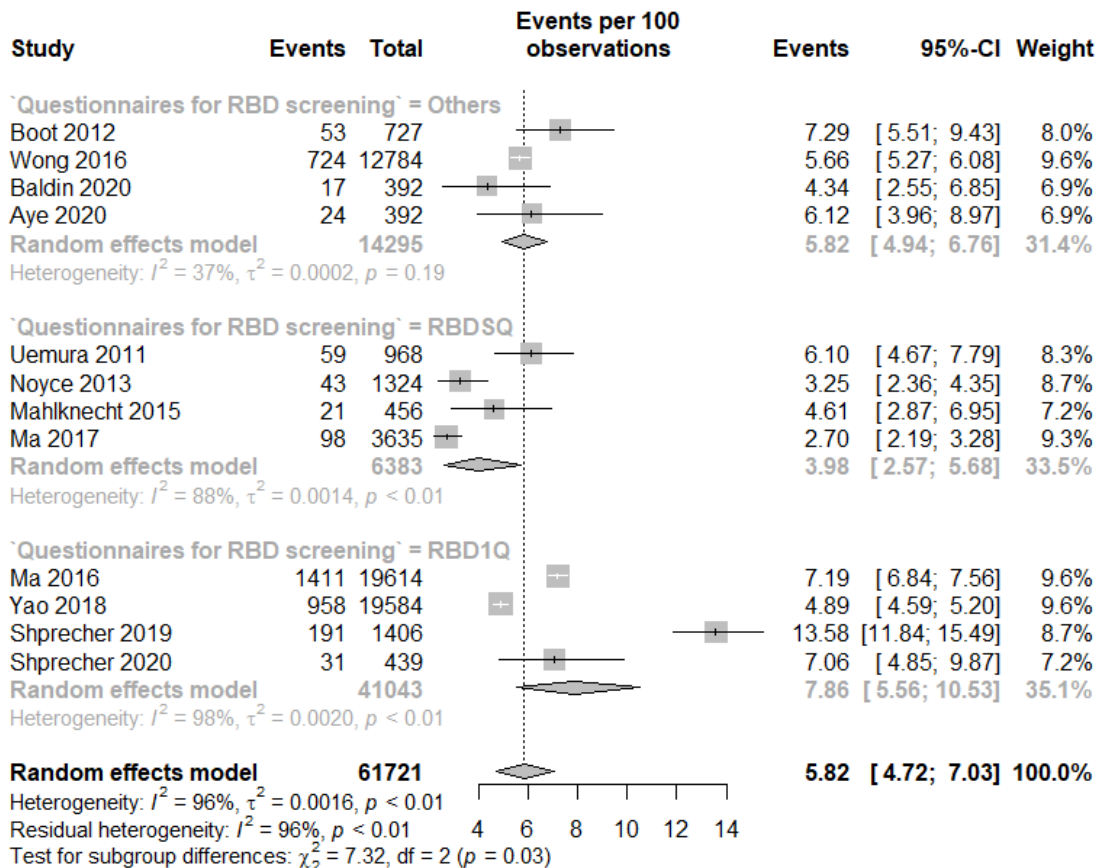geographic location.

Figure 6. Sensitivity analysis, pRBD studies classified according to the type of questionnaire used.

Legend. RBDSQ: RBD screening questionnaire; RBD1Q: RBD single question screen

Meta-regression analysis for pRBD

For pRBD studies, we found that the number of diagnostic stages ($R^2=19.5\%$; p=0.007) slightly explained the heterogeneity, while no other covariates explained

between-study heterogeneity: continent (R^2=0.0%), age range (R^2=0.0%), type of questionnaire (R^2=0.0%), sample size (R^2=0.0%) and quality of the studies (R^2=0.0%).

Discussion

Studying the prevalence of RBD is a challenging matter. The diagnostic criteria, in fact, require the PSG confirmation, that is difficult to perform, above all in population studies where often cases are classified only on clinical ground as pRBD. According to our results, the overall prevalence of PSG confirmed, definite RBD is 0.68%. However, when considering studies that did not use the PSG as confirmation, thus evaluating only pRBD, the pooled prevalence rate rose to 5.65%.

Prevalence of dRBD

Considering only the five studies with PSG confirmation, prevalence of dRBD ranges from 0.29% to 1.15%. Although these studies are considered the gold standard, the participation rate, that is around 50% in two out of five studies [2,22], represents the major limit biasing the estimation of the prevalence rate. Population studies are generally based on an active screening and it is possible to hypothesize that this approach leads to the identification of milder iRBD cases who experience

few or no consequence on their daily lives, not perceiving this condition as "medically relevant". On the contrary, in hospital-based studies, patients seek medical attention probably due to a more severe a form of iRBD. Few studies have formally investigated the differences between population-based and hospital-based iRBD patients and it has been found that iRBD cases identified in hospital setting presented a slightly worse cognitive performance [22,38] and a higher percentage of dream enactment behaviours (DEB) [22]. As a consequence, milder cases, when actively identified in population studies, are less prone to spend a night in hospital to perform PSG, explaining the low participation rate in some of the PSG studies. The exact accuracy of PSG at a very early stage of iRBD is not known, thus we cannot exclude that a certain percentage of early iRBD could have a negative PSG, especially if recorded just one time, possibly leading to un underestimation of the true prevalence. Furthermore, PSG is also limited by the so called "first night effect", that cannot be systematically accounted in population studies, where patients generally spend only one night in hospital to perform the examination. To the best of our knowledge only the HypnoLaus cohort study [21] reduced the risk associated to the first night effect by conducting the PSG recording with ambulatory devices in the subjects' homes. However, this study was the only one using a PSG recording without the use of video, thus possibly reducing the sensitivity of the procedure.

More stringent definition of iRBD could also play a role in the prevalence estimates. As such we have chosen to conduct a sensitivity analysis on the dRBD studies after excluding the study conducted by Chiu et al. [20], which assessed the prevalence of iRBD after screening for SRI, thus selecting only severe cases of iRBD. The prevalence estimates were close to the full model, and there was no significant heterogeneity among the studies, albeit I^2 was greatly reduced (5.98%) indicating a more homogeneous estimate.

Prevalence of pRBD

Due to the aforementioned limitations in conducting studies on dRBD, several questionnaire-based approaches have been tried in the general population, applying the definition of probable iRBD without a confirmatory PSG. Fourteen studies estimated the prevalence of pRBD reporting rates ranging from 0.57% to 13.58% giving a pooled prevalence of pRBD of 5.65% with a high level of heterogeneity (I^2= 98.55%).

Considering the geographical distribution, subgroup analysis found a borderline heterogeneity in the continent estimates that became significant after the removal of two outlying studies. Prevalence was lower for European studies (3.75%) with respect to both American (7.94%) and Asian studies (5.39%). Even if we have not a clear explanation, this difference can be explained by methodological differences

such as the smaller sample size of the three European studies [3,19,31] compared to the American [24,28-30] or Asian ones [23,25-27,32], rather than being linked to a true biological effect.

The quality of the studies has also impacted the estimates of pRBD, as demonstrated by a borderline significant heterogeneity between low- and high-quality studies, the latter expressing an overall lower prevalence (4.35%) compared to the former (7.60%). In fact, the six studies classified as low quality, generally did not describe the non-responders, except for one [30] nor provided the prevalence calculation. However, it is important to highlight that among these studies only one [30] explicitly studied the prevalence of iRBD, another focused on iRBD risk factors [26], while the majority of the studies focused on general parkinsonian signs [19,29,32,33].

Methodological differences were considered as possible modifiers of prevalence estimates. However, according to our analysis no significant heterogeneity was found considering both the sample size as well as for the age range of surveyed populations.

The type of questionnaires used is another source of possible heterogeneity, considering that their use as screening instruments is largely linked to their sensitivity and specificity. RBDSQ and RBD1Q are the two most used questionnaires. RBDSQ is composed by 10 questions with a sensitivity and

specificity for the diagnosis of RBD (using a cut-off ≥5) respectively of 96% and 92% [5]. RBD1Q has been validated in a multicentre study with a sensitivity of 93.8% and a specificity of 87.2% and is composed by a single question investigating the presence of abnormal movements during sleep [6]. The use of a single question makes this approach more feasible for population-based studies, however it also increases the risk of diagnosing as pRBD other RBD mimics. In fact, when applied on a general population, RBD1Q has demonstrated a very low positive predictive value (25%) [8]. Among questionnaires, only the Mayo Sleep Questionnaire [39] has been validated in a community-based sample but it has been used in only two studies [24,31]. In the sensitivity analysis, we found significant heterogeneity among the different questionnaires used with higher estimates for the RBD1Q [6] and lower for the RBDSQ [5].

We have also explored the impact on the prevalence estimates of the differences in study design and in particular one-stage or two-stage designs. Indeed, even if the Cochran Q test was not significant due to the low number of studies employing a two-stage design, prevalence rates were lower for the two-stage design (2.07%) approaching the prevalence estimates of dRBD studies. The two-stage approach reduces the number of false positives by correcting the high sensitivity of the screening questionnaires with the assessment conducted by a sleep specialist in a second stage, which can rule out some of the iRBD mimics after a clinical or telephonic examination. A three-stage design was also adopted in some studies in

which iRBD diagnosis was confirmed by PSG only in the group of highly suspected cases, thus in cases confirmed at the second stage after a clinical interview carried out by a sleep specialist [8,20,22]. This kind of study design represents the most effective one for iRBD epidemiological surveys. It allows for a large number of the general population to be surveyed through a validated questionnaire, with a second confirmatory stage only for a limited sample of subjects. Moreover, when PSG is available, the subjects positive to stage two can be invited to undergo PSG.

Even when we analyzed the source of heterogeneity through the meta-regression approach, none of the aforementioned modifiers significantly impacted the prevalence estimates.

The limits of our metanalysis are due to the low number of published studies, which limited the subgroup analysis in addressing some of the possible sources of heterogeneity.

However, it is the first study aimed at estimating the pooled prevalence of iRBD considering both the gold standard diagnosis and the pRBD diagnosis, highlighting some of the possible causes of differences in estimates in the two approaches.

The relevance of methodological precision in designing and conducting prevalence studies is of paramount importance, especially in the context of a rare disease, whose clinical manifestations are difficult to disclose and whose diagnostic confirmation is a complex issue. For iRBD, moreover, exact estimates are

extremely needed considering that it is the most promising candidate in future neuroprotective pharmacological trials addressing alphasynucleinopathies [40]. Consequently, future studies should employ a two or three stage approach and should survey larger populations, possibly in geographic areas where information on the RBD prevalence are still lacking, such as South America, North and Sub-Saharan Africa and Australia.

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3.0 Article 2

Prevalence of isolated RBD in the city of Catania, Italy: a population-based study

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Abstract

Study objectives: Few studies have analyzed the prevalence of isolated REM sleep Behavior Disorder (RBD) giving different estimates. Aim of the study was to estimate the prevalence of isolated RBD in the city of Catania.

Methods: A three-stage design was adopted. Participants attending the cabinets of General Practitioners in the city of Catania were screened with the RBD1Q questionnaire (Stage I). Positive participants were interviewed by phone and if suspected of RBD, were invited for clinical examination by a movement disorders specialist and a sleep specialist (Stage II). After the clinical examination, patients diagnosed as probable isolated RBD (pRBD) were invited to undergo a videopolysomnography (VPSG) (Stage III) to confirm the diagnosis of definite RBD (dRBD).

Results: A total of 1,524 participants have been screened. Of these, 220 (14.4%) screened positive. One-hundred-forty-three of them were further screened by phone, of whom 75 were suspected RBD. Thirty-six patients were diagnosed as pRBD giving a prevalence of 2.36% (95%CI 1.71-3.25). Twelve pRBD agreed to a VPSG and, of these, four were diagnosed as dRBD giving a prevalence of 0.26% (95%CI 0.07–0.67). Prevalence adjusted by non-participants was 3.48% (95%CI 2.67-4.52) and 1.18% (95%CI 0.45-1.37) for pRBD and dRBD respectively.

Conclusions: Prevalence of both pRBD and dRBD in Italy is comparable to the estimates reported in literature, confirming that isolated RBD has a low prevalence in the general population.

Introduction

Rapid Eye Movement sleep Behavior Disorder (RBD) is a condition characterized by the presence of abnormal behaviors in the REM sleep phase, such as sudden movements and vocalizations caused by a dream enactment behavior [1]. Definite diagnosis can be made only with a video-polysomnographic recording (VPSG) showing the lack of atonia during REM sleep and the presence of abnormal behaviors, according to the current diagnostic criteria[2].

Isolated RBD could be considered as an alpha-synucleinopathy in its earliest stages, conferring a high risk to convert to either Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB) or Multiple System Atrophy (MSA)[3]. Indeed, it represents the most specific risk factor for the development of PD being the strongest prodromal marker in the diagnosis of "Prodromal PD"[4], and is part of the core criteria for the DLB diagnosis[5]. To this reason, it is of paramount importance to study the prevalence of the disease in the general population. Nonetheless, data about prevalence of isolated RBD are scarce, with few population-based studies often reporting estimates that differ significantly depending on the diagnostic process employed [6,7].

According to a recent a recent meta-analysis, up to date five studies have evaluated the prevalence of isolated definite RBD (dRBD) (confirmed by VPSG) resulting in a pooled prevalence of dRBD of 0.68% (95%CI 0.38-1.05) while the pooled prevalence of probable RBD (pRBD, not confirmed by VPSG), based on 14 studies,

was 5.65% (95%CI 4.29-7.18)[8]. In Italy only two studies have been conducted on the prevalence of pRBD [7,9].

Aim of the current study is to assess the prevalence of both probable and definite isolated RBD in the city of Catania using a population-based three-stage design.

Methods

Study population and study design

The study has been conducted in the municipality of Catania, Italy (population: 314,555 inhabitants; ISTAT 2016) from April 2016 to November 2017.

For the first stage (screening phase – Stage I) a sample of general practitioners (GPs) working in the study area has been randomly selected from the provincial roster of the Italian Society of General Medicine to participate in the study. Before conducting the survey, several meetings have been carried out with the selected GPs to explain the aim of the survey. GPs have been given posters to be displayed in their waiting rooms explaining what is RBD.

Seven trained medical students visited each of the GPs' offices at least three times a week. Participants aged 40 years and above attended by the GPs have been face-to-face interviewed by the students who administered the RBD Single Question Screen (RBD1Q) questionnaire. The RBD1Q is a screening questionnaire with 94% sensitivity and 87% specificity validated in Italian language[10] and consists of the

following question: "Have you ever been told, or suspected yourself, that you seem to 'act out your dreams' while asleep (for example, punching, flailing your arms in the air, making running movements, etc.)?"

At the second stage (Stage II) participants positive at the screening phase underwent a structured phone-interview carried out by a board-certified sleep specialist (LG) in order to confirm the suspicion of isolated RBD. When the suspicion was confirmed, patients were invited to undergo a clinical evaluation at the Neurology Clinic of the "AOU Policlinico–Vittorio Emanuele" of Catania. A board-certified sleep specialist (LG) and a movement disorders expert (CEC) confirmed the presence of pRBD, based on a standardized semi-structured interview to exclude other sleep disorders and to evaluate the presence of other neurological disorders including parkinsonisms. When available, bed partners were contacted to provide information on the sleep behaviors. For all the enrolled patients at Stage II, the presence of extrapyramidal symptoms has been evaluated using the Unified Parkinson's Disease Rating Scale-III (UPDRS-III) [11]. In order to exclude patients with dementia at this stage, cognition and activities of daily living were assessed with the UPDRS sections I and II. Subjects with a suspected cognitive impairment underwent an extensive neuropsychological evaluation. The second stage allowed us to reach the diagnosis of probable isolated RBD (pRBD).

Patients considered as pRBD at second stage were invited to undergo a VPSG to confirm the presence of RBD (Stage III). Patients with a VPSG confirmed RBD

were diagnosed as definite isolated RBD (dRBD) according to the current diagnostic criteria[2]. In case of patients with a highly suggestive clinical history and presence of VPSG clinical events, but not satisfying the REM Sleep Without Atonia (RSWA) cut-off criteria for the diagnosis of RBD, a diagnosis of provisional RBD (provRBD) was proposed[2]. The study has been conducted in accordance with the STROND (Standards of Reporting of Neurological Disorders) guidelines[12].

Polysomnographic recordings

VPSG was recorded for at least one night for each subject. The VPSG recording was carried out using a minimum of eight-channel EEG, placed according to the International 10-20 system, two electrocardiographic derivations, submentalis muscle, the bilateral flexor digitorum superficialis muscle, and the bilateral anterior tibialis muscle electrodes, electro-oculogram, nasal thermistor, snore monitor, chest and abdominal movements, pulse rate and oximetry (Micromed SpA, Mogliano Veneto, Italy).

The VPSG recordings were scored by two investigators (LG, CEC) according to the AASM criteria[13] and, in case of disagreement, the conclusions were sorted out by discussion. RSWA was visually scored[13]. RBD was defined according to the ICSD-3 [2]. The presence of Periodic Limb Movements during Sleep (PLMS)

and Sleep Apneas was also recorded[13]. We considered pathological a PLMS index >15/h and an apnea/hypopnea index (AHI) >5/h[2].

Sample size calculation

Sample size calculation was based on a previous described prevalence of 0.74 %[14] in a European country with similar population characteristics to southern Italy. Thus, considering the population of the city of Catania in 2016, 0.5 precision interval and 95% Confidence Intervals (CI), a minimum number of 1,122 participants was calculated. Moreover, to account for an estimated proportion of 20% of non-participants, minimum sample size was increased to 1,346.

Statistical analysis

Demographic, clinical and instrumental data have been double entered in an ad hoc created database. Before analysis a range and consistency check has been conducted on the variables considered for the study. Missing data were identified and cross-referenced with the original documents. Qualitative variables have been described as frequencies while quantitative variables as means and standard deviations. Differences of demographic and clinical qualitative data have been analysed with the chi squared test and quantitative data with the t-test. When not-normally distributed appropriate non-parametric test have been used. Data have been

analysed with STATA 16.0 software. Lifetime prevalence and the 95% CI were calculated for isolated pRBD and dRBD. Moreover, the combined prevalence of provRBD and dRBD has been calculated. For pRBD age and sex specific prevalences have also been measured. Prevalence estimates considering only the population ≥50 and ≥60years have also been calculated. Prevalence rates for both isolated pRBD and dRBD have been adjusted projecting the obtained rates to the non-participants (both at Stage I and Stage II).

Ethics

The study has been approved by the Ethical Committee of the "AOU Policlinico-Vittorio Emanuele". All the patients have been given a paper briefly explaining the reasons of the study containing a written informed consent model to be signed.

Results

Stage I: screening phase

In the cabinets of 22 GPs who participated in the study, a total of 1,524 participants (642 [42.1%] men; mean age 62.2±11.7 years) were screened. Of these, 220 (14.4%) were positive at the screening questionnaire (mean age 63.8±11.6; 119 [54.1%] men). Flowchart of the participants at each of the study stages is reported in **Figure 1**. Participants who screened positive were older (p=0.03) and with a

higher prevalence of men (p<0.001). Demographic characteristics of the entire

sample are reported in **Table 1**.

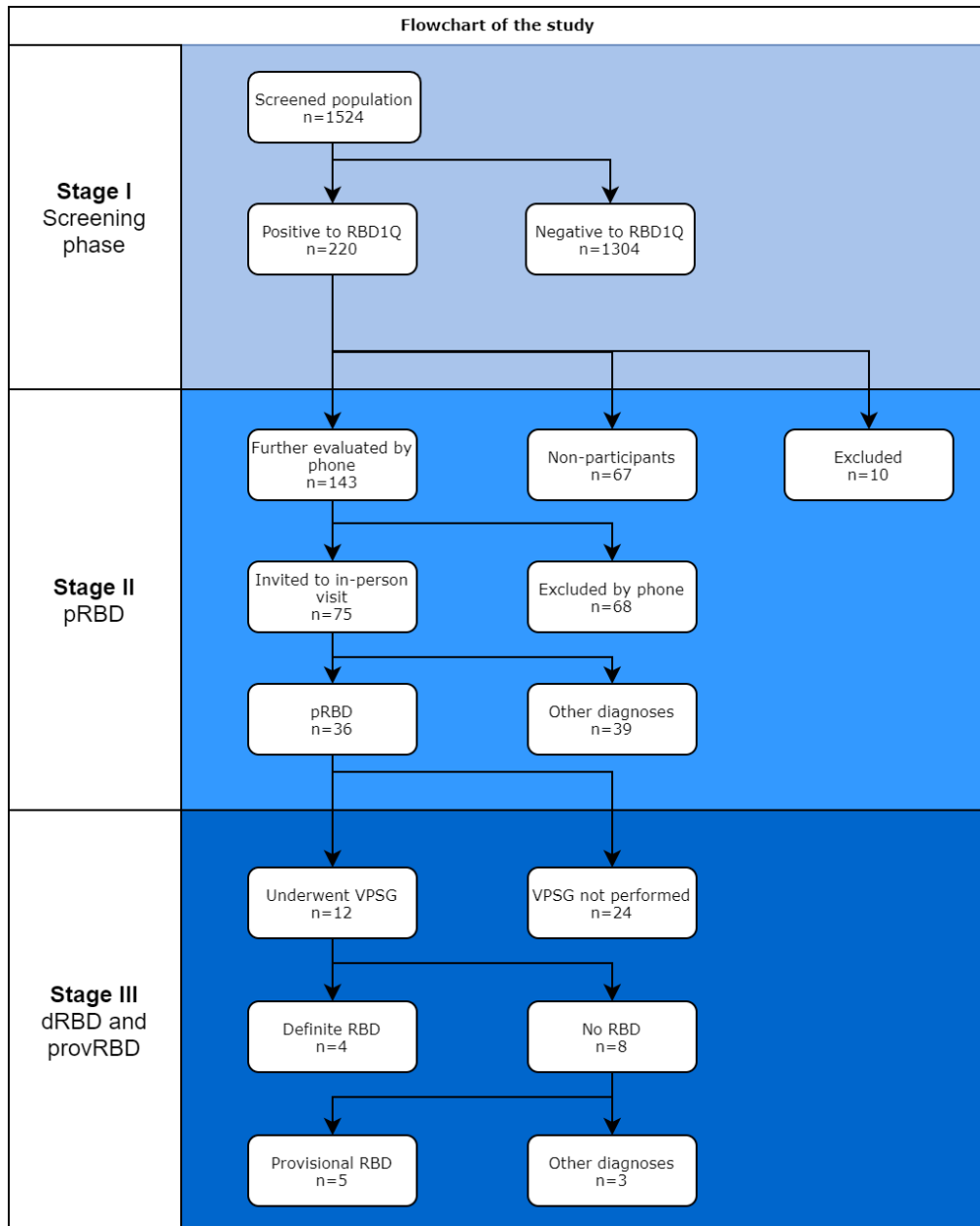**Figure 3.** Flowchart of the study.

Table 1 - Characteristics of the screened sample.

	Total N = 1524	Negative RBD1Q N = 1304	Positive RBD1Q N = 220	p value
Age, years (mean±SD)	62.2±11.7	61.9±11.7	63.8±11.6	**0.03**
Sex (Men), n(%)	642 (42.1)	523 (40.1)	119 (54.1)	**<0.001**
Marital status, n(%)				0.225
Not married	141 (9.3)	122 (9.4)	19 (8.6)	
Married	1111 (72.9)	958 (73.5)	153 (69.6)	
Widow	138 (9.1)	110 (8.4)	28 (12.7)	
Other	134 (8.8)	114 (8.7)	20 (9.1)	
Educational level, n(%)				0.086
Primary school	380 (24.9)	324 (24.9)	56 (25.5)	
Secondary school	544 (35.7)	461 (35.4)	83 (37.7)	
High school	370 (24.3)	327 (25.1)	43 (19.6)	
University	137 (9.0)	120 (9.2)	17 (7.7)	
Other	93 (6.1)	72 (5.5)	21 (9.6)	
Occupation, n(%)				**0.021**
Unemployed	121 (7.9)	100 (7.7)	21 (9.6)	
Employee	255 (16.7)	218 (16.7)	37 (16.8)	
Housewife	442 (29.0)	400 (30.7)	42 (19.1)	
Professional	87 (5.7)	72 (5.5)	15 (6.8)	
Retired	515 (33.8)	428 (32.8)	87 (39.6)	
Other	104 (6.8)	86 (6.6)	18 (8.2)	
Familiar history for Parkinson's Disease, n(%)	51 (3.4)	39 (3.0)	12 (5.5)	0.06

Stage II: prevalence of isolated pRBD

Of the 220 who screened positive, 10 (4.5%) were excluded because they were either deceased (n=6) or did not meet inclusion criteria (three had a neurodegenerative disease and one a demyelinating disease). Sixty-seven (31.9%) out of the 210 screened positive did not participate at Stage II (29 [43.2%] could

not be traced and 38 [56.7%] refused to participate in the study). Finally, 143 participants have been further evaluated leading to a participation rate at Stage II of 68.1% (**Figure 1**). Compared to non-participants, those who have been evaluated were younger (mean age 61.9±11.4 vs 66.3±11.0; p=0.01) and with a higher educational level (high school graduated 23.8% vs 10.5%; p=0.02).

Sixty-eight of the 143 (47.5%) screened positive were excluded because the suspicion of RBD was not confirmed at the phone interview. Out of the 75 suspected RBD 73 were in-person evaluated at the Neurologic Clinic while two were unable to come to the hospital and the diagnosis of pRBD was confirmed just by an accurate phone-interview.

Of the 75 suspected RBD, 53 (70.7%) had also a bed partner that shared information on the nocturnal behaviors of the patients. Thirty-nine (52%) were excluded because seven (18.0%) had a suspicion of NREM parasomnia, six (15.4%) of Restless Leg Syndrome (RLS), 10 (25.6%) of insomnia, six (15.4%) of Obstructive Sleep Apnoea (OSA) while three (7.7%) presented other alternative diagnoses such as post-traumatic stress disorder, epileptic seizures and laryngospasm, and four (10.2%) a sleep complaint of uncertain clinical significance. Further three patients (7.7%) were excluded because presented other associated disorders thus leading to a diagnosis of secondary RBD (two PD and one DLB).

Finally, 36 patients (20 men [55.6%]; mean age 62.5±10.8 years) fulfilled the diagnosis of isolated pRBD giving a prevalence of 2.36% (95%CI 1.71-3.25); a

similar prevalence of 2.45% (95%CI 1.73-3.46) was obtained for the population aged ≥50 years and 2.53% (95%CI 1.66-3.84) for those aged ≥60. Prevalence was higher among men (3.10%; 95%CI 2.01-4.74) than women (1.82%; 95%CI 1.12-2.93) and steeply increased with age starting from 1.91% (95%CI 0.82-4.40) in the population aged 40-49 years to reach a peak of 3.38% (95%CI 1.98-5.70) in the group aged 60-69 and to slowly decline soon after (**Table 2)**. Baseline characteristics of pRBD are reported **in Table 3**.

Table 2 - Age and sex specific prevalence of pRBD.

Age classes	Men			Women			All		
	Sample	Cases	Prevalence (95% CI)	Sample	Cases	Prevalence (95% CI)	Sample	Cases	Prevalence (95% CI)
40-49	93	2	2.15% (0.60-7.50)	168	3	1.78% (0.61-5.11)	261	5	1.91% (0.82-4.40)
50-59	146	5	3.42% (1.47-7.76)	262	5	1.91% (0.82-4.38)	408	10	2.45% (1.33-4.45)
60-69	177	5	2.82% (1.21-6.44)	207	8	3.86% (1.97-7.43)	384	13	3.38% (1.98-5.70)
70-79	165	6	3.63% (1.67-7.70)	200	0	0	365	6	1.64% (0.75-3.53)
80-89	61	2	3.27% (0.90-11.19)	42	0	0	103	2	1.94% (0.53-6.80)
90-99	3	0	0	0	0	0	3	0	0
Total	645	20	3.10% (2.01-4.74)	879	16	1.82% (1.12-2.93)	1524	36	2.36% (1.71-3.25)

Legend: CI, confidence interval; pRBD, probable RBD.

Table 3 - Clinical and demographic characteristics of patients with pRBD, dRBD and provRBD.

	pRBD N = 36	dRBD N = 4	provRBD N = 5
Age, years (mean±SD)	62.5±10.8	65.2±12.5	52.5±8.5
Age, years (median and range)	63 (42-86)	64 (52-81)	54 (42-62)
Sex (men), n(%)	20 (55.6)	2 (50)	2 (40)
UPDRS-III	5.7±5.1	4.5±4.5	4±2.3
Sleep macrostructure			
Total Sleep Time, min (mean±SD)	\	323.7±71.9	314.2±114.2
Sleep latency, min (mean±SD)	\	34±19.2	7.4±7.3
Sleep efficiency% (mean±SD)	\	77±7.2	83.8±8.6
Wake after sleep onset, min (mean±SD)	\	84±23.3	59.2±30.3
N1% (mean±SD)	\	8±4.3	2.6±3.2
N2% (mean±SD)	\	52±11.0	52±9.9
N3% (mean±SD)	\	24.8±7.4	24.6±12.5
REM% (mean±SD)	\	16.3±3.4	19.2±7.5

Legend: pRBD, probable RBD; dRBD, definite RBD; provRBD, provisional RBD; N, number; SD, Standard Deviation; min, minutes; UPDRS-III, Unified Parkinson's Disease Rating Scale-III.

Stage III: prevalence of isolated dRBD

Out of the 36 patients with pRBD only 12 (33.3%) agreed to spend a night in the Clinic to undergo a VPSG. The 24 (66.7%) pRBD who refused the VPSG were slightly older (65.8±9.4 vs 56.4±11.3; p=0.01), but apart from age, no significant differences were found. Considering the 12 patients who underwent VPSG, four (33.3%) were diagnosed as isolated dRBD, while five (41.7%) were diagnosed

as provRBD, reaching a confirmation rate of 75% (**Figure 1**). For the other three patients diagnoses of OSA, RLS and fragmented sleep were made. Among the dRBD cases two also presented a PLMS index >15/h, while one showed an AHI higher than 5/h. Sleep comorbidities for provRBD were PLMS in two and sleep apnea in one. provRBD cases were slightly younger when compared to dRBD (**Table 3**).

Considering the four patients with isolated dRBD prevalence was 0.26% (95%CI 0.07–0.67) with a slightly higher prevalence among men (0.31% [95%CI 0.04-1.0] *versus* 0.23% [0.006-0.8]). Prevalence of dRBD was slightly higher both in the population aged ≥50 (0.31% [95%CI 0.12-0.81]) and in the population aged ≥60 years (0.36% [95%CI 0.12-1.06]). Prevalence reached 0.59% (95%CI 0.27-1.12) when also provRBD cases were considered. Clinical, demographic and polysomnographic characteristics of patients with dRBD and provRBD are reported in **Table 3.**

Adjusted prevalence of isolated pRBD and dRBD

When prevalence rates obtained for participants have been applied to non-participants, prevalence of pRBD was of 3.48% (95%CI 2.67-4.52) while prevalence of dRBD was 1.18% (95%CI 0.45-1.37). Considering both dRBD and provRBD prevalence adjusted by non-participants was 2.62% (95%CI 1.93-3.55).

Discussion

Compared to other neurologic diseases, there is still paucity of information on the epidemiology of RBD. According to a recent metanalysis the pooled prevalence of dRBD is 0.68% and 5.65% for pRBD[8]. However, prevalence estimates vary widely across the studies due to the different methodological approaches, study designs, diagnostic criteria, screening questionnaires adopted, age structure of the selected populations and participation rate[8].

In our study, using a three-stage design, we found a prevalence for of isolated pRBD of 2.36% and 0.26% for dRBD that rose up to 0.59% when patients with provRBD were considered. Our prevalence estimates are lower than those reported in the metanalysis on isolated RBD, albeit within the observed range for both dRBD and pRBD diagnosis[8]. Nonetheless, they get closer to those reported in literature when adjusted by the participation rate.

Prevalence of isolated pRBD: Stage II

In our study, at stage two, we found a prevalence of pRBD of 2.36%. However, 30.5% of the screened population did not participate at the second stage and when prevalence rate was adjusted projecting the observed rate to the non-participants, an adjusted prevalence rate of 3.48% was obtained.

Up to date 14 studies have evaluated the prevalence of pRBD and rates reported ranges from 0.6% to 13.6%[8]. Although our prevalence is in the range of those reported in literature it is lower than the average pooled prevalence (5.65%). However, the majority of these studies adopted a one-stage design, thus the diagnosis of pRBD was not confirmed through a clinical interview but was only based on the screening questionnaires. Comparison with these studies is in general difficult, because one-stage studies tend to overestimate the prevalence of RBD (pooled prevalence 6.40%)[8], that in this case depends on the sensitivity and specificity of the adopted questionnaires. Indeed, sensitivity and specificity of RBD screening questionnaires depend on the studied population[15], clinical setting[16] and might not be consistent across repeated evaluations[17]. Except for the Mayo Sleep Questionnaire[18], the majority of these tools have been validated just in a hospital setting. Nonetheless, it is well known that hospital validations tend to overestimate both sensitivity and specificity levels[19]. Furthermore, the one-stage design does not allow to exclude the presence of secondary RBD, such as RBD associated to alpha-synucleinophaties.

Only two studies adopted a two-stage design in which participants who screened positive were confirmed by a clinical evaluation (Stage II)[7,20]. Prevalence rates reported in these latter studies were on average lower with respect to the one-stage studies (pooled prevalence 2.1%)[8] and closer to our estimates.

To the best of our knowledge only two small studies involving about 400 participants aged 60 years and above, evaluated the prevalence of pRBD in Italy[7,9]. In particular a two-stage study was carried out in the Trentino-Alto Adige region[7] and reported a prevalence of 4.6%, while a one-stage survey, focused on the mild parkinsonian signs, was carried out in the Emilia Romagna region[9] and reported a prevalence of 4.3%. These estimates are higher with respect to the rate reported in our study (2.36%), but close if we consider the adjusted rate (3.48%). The participation rate at Stage II (68.1%) in our study could, in fact, in part explain such a difference, but we strongly believe that the confirmation of the pRBD at Stage II by an expert on sleep disorders has played an important role in lowering the number of false positive. As a matter of fact, only 16.4% of the screened positive at Stage I has been confirmed at Stage II. In agreement with other studies prevalence of pRBD was higher among men[20,22].

Prevalence of isolated dRBD: Stage III

Our study is the first VPSG based study on the prevalence of isolated RBD in Italy. Prevalence of isolated dRBD in our study was 0.26% but reached 0.59% when provRBD were also considered. These rates are close to those reported in literature[8]. Only five studies aimed to determine the prevalence of isolated dRBD have been carried out reporting rates ranging from 0.29% to 1.15% (pooled 0.68%)[8] and of these, three adopted a similar three-stage design and

reported on average a low prevalence rate ranging from 0.29% to 0.74%[14,23,24].

In particular, our prevalence for dRBD (0.26%) is lower than that reported by a European three-stage study performed in Spain[14] where prevalence of dRBD was of 0.74%. However, the participation rate in our study at both stage Stage II (68.1%) and Stage III (33.3%) was lower than that reported in the Spanish study. Indeed, when adjusting for the non-participants, prevalence of dRBD in our sample was closer to the Spanish one (1.18%). Another similar three-stage survey has been carried out in Japan where a prevalence of dRBD of 0.54% was reported[23]. In this latter study prevalence rose up to 1.23% when also provisional RBD was considered. This estimate is close to that obtained in our survey when patients with provRBD have been included. Interestingly, the adjusted prevalence rates of dRBD (1.18%) and dRBD plus provRBD (2.62%) in our study were almost double, when compared to this study, where the participation rate was slightly above the 50%[23].

Finally, a third three-stage survey was carried out in China where, considering only isolated dRBD, prevalence was 0.29%[24]. Comparison with the other two studies is limited because of different inclusion criteria and procedures[6,25].

Weaknesses and strengths

Our survey confirms that isolated RBD is a disease with a very low prevalence rate in the general population, considering both pRBD and dRBD and underlines the difficulties in carrying out population-based surveys above all for dRBD.

One of the main pitfalls in conducting a prevalence survey to estimate RBD prevalence is related to the participation rate, especially for the diagnosis dRBD that requires the VPSG recording.

Participation rates, across the different stages, vary widely between studies, with some having low participation rates[20,23] and other higher[14,24,25]. In our survey the participation rate was good (almost 70%) for the Stage II (clinical evaluation), but very low (33%) at Stage III (VPSG examination). In particular, participation rate at Stage III was lower than that recorded in the Spanish study[14], but higher when compared to a Japanese study where none of the participants agreed to a VPSG[20]. The issue of participation rate has a relevant impact in interpreting prevalence estimates since the low participation rate can lead to an underestimation of the true prevalence when rates are not adjusted by the number of non-participants and can also lead to a selection bias limiting the generalizability of the results.

There are different factors that might have contributed to the low participation rate at the VPSG examination. First, patients affected by RBD are often not aware about their disorder, that is usually considered as a paraphysiological behavior. To this reason they often do not agree to spend a night at the hospital to undergo

VPSG. Another cause for refusal reported by the enrolled participants was related to the fact that they have been informed about the possible association between RBD and PD and, to this reason, preferred to avoid further investigations. Indeed, ethical issues in RBD epidemiological studies are a delicate matter balancing between advantages and disadvantages of disclosing alpha-synucleinopathy risk information in such research settings[26]. In our study we chose an approach based on full disclosure of the scope of the study, explaining also the associated risk of developing an alpha-synucleinopathy, albeit underlining that the real extent of the risk is not well understood.

A further important limit in interpreting the estimates of dRBD reported in literature is related to the VPSG procedure. The rate of pRBD confirmed by VPSG is generally very low[14]. Indeed, if on one hand the diagnosis based on clinical grounds (pRBD) tends to be overestimated, on the other one the true prevalence of dRBD confirmed by VPSG could be underestimated. VPSG, in fact, might fail to capture the presence of RBD because of the first night effect and, more importantly, because of the non-persistence of RBD symptomatology through every night[27], especially when patients are actively screened in a population-based setting, where symptoms are deemed so mild that they are not considered worthy of medical assistance. From this point of view, the inclusion of provRBD could be important in reducing the number of false negative cases.

We also acknowledge that our prevalence estimates could be lower than the true population prevalence of RBD due to several pitfalls. Indeed, non-participants at Stage I were older and less educated when compared to participants, both considered factors associated with RBD[28]. Moreover, participants sleeping alone might not be aware of mild movements during sleep, and thus not considering having a sleep disorder, reducing the prevalence estimates. Furthermore, it has been demonstrated[6], that a certain portion of patients without a clear evidence of movements during sleep may just present features of RSWA (isolated RSWA)[6]. On this ground a VPSG should be performed in a random sample of the screened negative participants, in order to estimate the percentage of false negative on the bases of the clinical history, but these kind of studies are difficult to perform. Indeed, considering the low prevalence of RBD, a large number of negative subjects should undergo VPSG in order to obtain accurate estimates. Nevertheless, it should be noted that the presence of isolated RSWA is not sufficient to diagnose RBD[2], and thus even these results should be interpreted with caution.

Finally, another potential source of selection bias is represented by the sampled populations. In our study the selected population has been drawn from the GPs offices. Considering the characteristics of the study outcome, door-to-door design could be a better approach, but it is extremely expensive and time consuming, thus poorly feasible. On the other hand, a study design including participants

admitted for general consultation in their local GPs studies represent a good compromise, as other studies have already done[9,14]. Nonetheless, even if we randomly selected a sample of GPs working in the city of Catania, participants enrolled were those who attended the GP's offices, thus we cannot be sure that they were truly representative of the study population.

However, regardless of the above-mentioned limits, our study has many strengths of which the large sample size and the three-stage design represent the main ones. This is, in fact, the first survey conducted in Italy, and one of the largest, to determine the prevalence of isolated dRBD, using a population-based design. The size of the study was determined according to a specific sample size calculation and, in order to obtain a representative sample of the population GPs were randomly selected from the roster of the province of Catania. A further important strength is related to the confirmation of pRBD by a certified sleep specialist, that allowed us to reduce the number of false positive from Stage I to Stage II and to correctly classify the other sleep disorders reported by non-RBD cases at Stage II. As consequence, on one hand, a lower prevalence pRBD has been detected, and on the other hand, the confirmation rate at Stage III was quite high when dRBD and provRBD were considered. Furthermore, patients with suspicion of RBD at Stage II were also extensively evaluated by a movement disorders specialist able to recognize also early stage of alpha-synucleinopathies and to correctly apply the diagnostic criteria in order to exclude secondary RBD.

In conclusion our study confirms that isolated RBD is a disease with a low prevalence and has underlined important limits in carrying out population-based surveys to detect dRBD. From an epidemiological point of view surveys aimed to estimate the isolated pRBD are more feasible, even if this kind of studies could lead to an overestimation of the outcome. The two-stage design and the use of sleep specialists to confirm pRBD cases represent an important requirement to reduce the number of false positive patients. Considering that isolated RBD is considered the best "window of time" to test a potential neuroprotective drug that might hinder or stop the progression to an alpha-synucleinopathy[29], epidemiological guidelines to perform surveys on RBD prevalence are needed in order to obtain more homogeneous estimates.

Acknowledgments

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4.0 Article 3

Retinal thickness and microvascular pathway in Idiopathic Rapid eye movement sleep Behaviour Disorder and Parkinson's disease.

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Abstract

Introduction: Retinal impairment has previously been described in Parkinson's Disease (PD), also in early stage of disease. Idiopathic Rapid-eye-movement sleep Behavior Disorder (iRBD) is considered the strongest marker in the diagnosis of "Prodromal PD". Thus, we evaluated the thickness of retinal layers and the microvascular retinal pattern in a group of iRBD patients compared to PD and healthy subjects (HCs).

Methods: retinal layer's thickness and microvascular pattern among PD, iRBD and HCs were assessed using Spectral-Density Optical Coherence Tomography (SD-OCT) and OCT-Angiography (OCT-A), respectively.

Results: Forty-one eyes from 21 PD, 37 eyes from 19 iRBD and 33 eyes from 17 HCs were analyzed. Peripapillary Retinal Nerve Fiber Layer (RNFL) was thinner in PD and RBD compared to HCs. All macular retinal layers, except for retinal pigment epithelium, resulted to be significantly thinner in iRBD and in PD compared to HCs, also adjusting by age, sex and hypertension. Macular RNFL and ganglionic cell layer were thinner in PD compared to iRBD. Moreover, in iRBD, a peculiar microvascular pattern was found, characterized by a higher vascularization of the deep capillary plexus with respect both PD patients and HCs.

Conclusion: in PD and iRBD patients retina was thinner than HCs, and values of iRBD were between PD and HCs. Moreover, in iRBD, a peculiar microvascular

pattern has been found, characterized by a higher vascularization of the deep capillary plexus. Our findings suggest that retina might be considered a biomarker of neurodegeneration in iRBD, easily estimable using non-invasive tool such as OCT and OCT-A.

Introduction

Idiopathic Rapid eye movement sleep Behavior Disorder (iRBD) is a condition characterized by the presence of abnormal behaviors in the REM sleep phase, such as movements and vocalizations caused by a dream enactment behavior[1]. In the last decades, increasing evidences from epidemiological studies have shown that presence of iRBD is associated with a higher risk of developing a neurodegenerative disease, especially α-synucleinopathies such as Parkinson's Disease (PD), Dementia with Lewy Bodies (DLB) and Multiple System Atrophy (MSA)[1]. In a recent multicenter study of iRBD patients, up to 28% of the sample converted to an α-synucleinopathy with a mean time to phenoconversion of 4.6 years[2]. Previous studies with longer follow-up demonstrated that up to 90% of patients developed a neurodegenerative disease almost 14 years after RBD diagnosis[3]. On the bases of these evidences iRBD is considered the most specific risk factor for the development of PD, being the strongest marker for the diagnosis of "Prodromal PD"[4].

Although PD has been classically considered a movement disorder, several non-motor symptoms represent very common features of the disease[4]. Among NMSs, visual impairment, including color vision, visual acuity and contrast sensitivity[5] has been also described in an early stage of PD[5]. Furthermore, several studies based on Optical Coherence Tomography (OCT) evaluation, have reported a lower retinal thickness among PD patients as compared to healthy

subject[6]. In agreement with these observations we have recently described a thinning of inner retinal layers in PD patients at early stage of disease[7]. Moreover, we have also reported a positive correlation between inner retinal thickness and microvascular density in the foveal region, known to be involved in visual acuity and colour vision[7].

Visual impairment has been reported in 40-60% of iRBD subjects, especially colour vision dysfunction[8,9] and the risk of conversion to parkinsonian clinical forms seems to be higher among iRBD with abnormal colour discrimination[2,9]. On this ground retinal impairment could be part of the neurodegenerative process and it could be present also during the prodromal phase of PD, before the motor onset. To the best of our knowledge, only one study recently evaluated macular retinal thickness using retinal segmentation analysis, reporting a thinning of ganglion cell complex (GCC) in iRBD as compared to healthy controls (HCs)[10]. Unlike PD, up to date no studies investigated the possible presence of microvascular impairment already in the prodromal phase of PD, such as in iRBD.

Thus, in this study, we evaluated the thickness of retinal layers and the microvascular retinal pattern in a group of iRBD patients compared to PD and healthy subjects using, respectively, OCT and Spectral-Domain OCT angiography (OCT-A).

Materials and methods

Study population

Three groups of subjects were enrolled: PD patients, iRBD patients and HCs.

We analysed PD patients enrolled in our previous study[7]. In detail, early PD patients attending the "Parkinson's Disease and Movement Disorders Centre" of the University of Catania and fulfilling the MDS-PD diagnostic criteria for clinically established or clinically probable PD were enrolled. [7,11].

iRBD were enrolled among both subjects previously identified in a population-based study investigating iRBD prevalence in the community of Catania[12] and patients attending to the Clinic of Neurology of the University of Catania. Diagnosis of definite iRBD (dRBD) was sought on the base of a video-polysomnographic recording (VPSG), showing the lack of the physiological atonia during REM sleep phase associated to abnormal motor behaviors or vocalizations, scored according to the American Academy of Sleep Medicine (2014). When VPSG was not available, confirmation of the presence of RBD was performed by a board certified sleep expert (LG) using a semi-structured interview based on the RBD screening questionnaire (RBDSQ)[13]. These patients were diagnosed as probable iRBD (pRBD)[14].

A group of HCs was selected from caregivers of PD and iRBD patients attending our centre. All controls underwent a neurological examination by trained neurologists, to exclude any neurological disease.

Neurological examination was performed by neurologists expert in movement disorders. Motor impairment was evaluated with the Unified Parkinson's Disease Rating Scale part-III (UPDRS-III) and the Hoehn and Yahr (HY) scale. For PD patients clinical and pharmacological data were collected from patient's medical records.

Ophtalmologic evaluation

All subjects underwent a complete ophthalmologic examination, performed at "Ophthalmology Clinic" of University of Catania. It included a standardized clinical examination with the assessment of visual acuity, intraocular pressure (IOP), fundus examination, slit-lamp biomicroscopy, OCT and OCT-A. We excluded all subjects with a history of ocular trauma, ocular surgery, ocular diseases involving retina, optic nerve, cornea or macula, IOP>21 mmHg, cataract, systemic condition that could impair visual system, such as diabetes mellitus, uncontrolled hypertension or hypotension, cardiovascular diseases and any other neurological disease.

High-definition Optical Coherence Tomography (HD-OCT) and Spectral-Domain Optical Coherence Tomograph-Angiography (SD-OCT-A) imaging

Macular retinal thickness and peripapillary retinal nerve fiber layer (RNFL) thickness were assessed using the Cirrus HD-OCT model 5000 (Carl Zeiss

Meditec, Inc). SD-OCT-A of the macula and peripapillary plexus were performed using AngioVue XR Avanti (Optovue Inc, Fremont, California, USA). OCT and OCT-A protocols have been extensively reported elsewhere[7]. In brief, following layers were analysed: RNFL, Ganglionic Cell Layer (GCL), Inner Plexiform Layer (IPL), Inner Nuclear Layer (INL), Outer Plexiform Layer (OPL), Outer Nuclear Layer (ONL), Retinal Pigment Epithelium (RPE). AngioVue automatically segments the area into four layers, including superficial capillary plexus layer (SCP), deep capillary plexus layer (DCP), that are, in turn, subdivided into foveal, parafoveal, superior and inferior area.

Data collection

Retinal images of both left and right eye were acquired for each subject. The quality of each ocular image was evaluated by expert ophthalmologists (MR,AL,AR,NC) and eyes whose ocular measurements were not of good quality were excluded from the analysis. All collected data have been entered in ad hoc created database using Excel software and each subject was identified using a unique identification code to protect anonymity. Before analysis, a consistency check has been performed for all the variables in the database.

Statistical analysis

Data were analyzed using STATA 12.1 software (StataCorp, College Station, TX, United States). Quantitative variables were described using mean and standard deviation. The difference between means was evaluated by the t-test and ANOVA-test while the difference between proportions by the Chi-squared test. In case of a not normal distribution, appropriate non-parametric tests were performed. To evaluate the possible association between iRBD and the thickness of each retinal layer an unconditional logistic regression analysis was performed. Multivariate analysis was performed considering age and sex as *a priori* confounders. Moreover, considering the influence of blood pressure values on microvascular pattern, all results were adjusted also by presence of hypertension on medical history, using multivariate model. The odds ratios (OR) with 95% confidence intervals (CI) and p value (two-tailed test, $\alpha=0.05$) were calculated. Pearson correlation analysis was performed to evaluate the presence of correlation between retinal layers thickness and microvascular pattern. These data were also adjusted for age, sex and hypertension. The significance level was set at 0.05 and the ninety-five confidence intervals (95% CI) were calculated.

Standard Protocol Approvals, Registrations and Patient Consents

This study was carried out in accordance with Declaration of Helsinki and approval from the local ethical committee (Ethical Committee Catania 1) was

obtained. All the participants have been asked to sign an informed consent prior to be included in the study.

Results

Descriptive analysis

Twenty-one PD patients [12 men, 57.1%; age (means ± SD) 61.5 ± 6.5], 19 iRBD subjects [11 men, 57.9%; age 58.8 ± 13.3 years] and 17 HCs [9 men, 52.9%; age 65.1 ± 10.7] were enrolled in the study. Age and sex were not significantly different across the three groups (Table 1).

Table 1. Demographic and clinical characteristics.

	PD n. 21 (n. 41 eyes)	iRBD n. 19 (n. 37 eyes)	HCs n. 17 (n. 33 eyes)	p-value * iRBD vs PD	p-value * iRBD vs HCs
Men, *n (%)*	12 (57.1%)	11 (57.9%)	9 (52.9%)	0.96	0.76
Age at OCT *(years)*	61.5±6.5	58.8±13.3	65.1±10.7	0.41	0.14
Age at onset *(years)*	59.3±7.0	/	/	/	/
Disease duration *(months)*	27.4±14.3	/	/	/	/
HY stage	1.9±0.4	/	/	/	/
UPDRS-ME score	25.0±6.9	5.6±4.3	3.2±2.7	**<0.001**	0.07
Education *(years)*	10.5±3.3	9.6±2.9	11.0±3.7	0.37	0.22
LD, *n (%)*	11 (52.4%)	/	/	/	/
LED *(mg)*	127.4±142.7			/	/
	12 (57.1%)	4 (21.1%)	4 (23.5%)	**0.02**	0.86

Hypertension, *n (%)*		

Legend: PD (Parkinson's disease); iRBD (Idiopathic Rapid eye movement sleep Behavior Disorder); HCs (healthy controls);

HY (Hoehn and Yahr scale); UPDRS-ME (Unified Parkinson's Disease Rating Scale part III);; LD (Levodopa); LED (Levodopa Equivalent Dose).

* Using t-test.

PD patients were at an early stage of disease, with a mean duration from the clinical onset to the neurological evaluation of 27.4 ± 14.3 months (disease duration). History of hypertension was significantly more frequent among PD patients as compared to both iRBD and controls (Table 1).

Among the enrolled iRBD subjects, 9 (47.4%) underwent a VPSG and they were all diagnosed as dRBD (4 from the population-based study and 5 from patients attending to our Clinic of Neurology), while the others were considered as pRBD. There were not significant differences in age, sex and UPDRS-III score between probable and definite RBD (Supplementary table 1).

Supplementary table 1. Demographic and clinical characteristics of pRBD and dRBD groups.

	dRBD n.9 (n. 17 eyes)	pRBD n.10 (n. 20 eyes)	p-value
Men, *n (%)*	6 (66.7%)	5 (50%)	0.46
Age (*years*)	63.8±15.2	54.4±10.0	0.13
UPDRS-ME score	7.1±5.5	4.3±3	0.21
Education (*years*)	8.5±3.1	10.5±2.6	0.16
Hypertension, *n (%)*	3 (33.3%)	1 (10%)	0.21

Legend: dRBD: definite RBD; pRBD: probable RBD; UPDRS-ME: Unified Parkinson's Disease Rating Scale-Motor Examination

OCT analysis–Comparison of macular retinal layers thickness among PD, iRBD and HCs

A total of 41 eyes from 21 patients with PD, 37 eyes from 19 subjects with iRBD and 33 eyes from 17 HCs were analysed using OCT. One eye from PD patients, 1 eye from iRBD subjects and 1 eye from HCs were excluded because of the poor quality of OCT.

The thickness of each retinal layer was not significantly different between right and left eye in PD, HCs, and iRBD groups. Thus, data from both eyes were considered to perform statistical analysis. Due to the lack of statistically significant differences in the thickness of each retinal layer between pRBD and dRBD, iRBD subjects were analysed considering a single group (Supplementary table 2).

Supplementary table 2. Thickness of retinal layers in pRBD and dRBD.

	dRBD n.9 (n. 17 eyes)	pRBD n.10 (n. 20 eyes)	p-value
RNFL	15.2±1.8	15.6±1.8	0.43
GCL	18.3±2.9	20.0±2.2	0.05
IPL	22.9±1.8	22.1±3.4	0.37
INL	24.5±2.9	22.2±4.4	0.08
OPL	27.8±2.9	26.5±3.7	0.26
ONL	86.5±5.5	83.5±11.2	0.32
RPE	16.1±1.0	16.1±1.2	0.96
NOG	98.3±8.9	95.7±9.6	0.41
NOG SUP	113.5±8.6	115.9±11.1	0.46
NOG TEMP	71.9±7.5	73.7±9.6	0.52
NOG INF	118.1±7.9	120.1±10.3	0.52
NOG NAS	79.9±8.8	79.1±8.4	0.80

Legend: dRBD: definite RBD; pRBD: probable RBD; RNFL: Retinal Nerve Fiber Layer, GCL: Ganglionic Cell Layer, IPL: Inner Plexiform Layer, INR: Inner Nuclear Layer, OPL: Outer Plexiform Layer, ONL: Outer Nuclear Layer, RPE: Retinal Pigment Epithelium, G ON: Global Optic Nerve, SUP ON: Superior sector - Optic Nerve, TEMP ON: Temporal sector - Optic Nerve, INF ON:

Inferior sector - Optic Nerve, NAS ON: Nasal sector - Optic Nerve. * Using t-test, to compare two groups.

The thickness of macular RNFL, GCL, IPL, INL, OPL, ONL was statistically different across the three study groups (Table 2). In particular, all retinal layers, except for RPE, have been found to be significantly thinner in iRBD patients compared to HCs and, even more, in PD patients (Table 2). Comparing PD patients and iRBD subjects, RNFL and GCL resulted to be significantly thinner in PD, while significant differences were not found in the other layers (Table 2).

Table 2. Thickness of retinal layers in PD, iRBD and HCs group, assessed by OCT segmentation analysis.

	PD n. 21 (41 eyes)	iRBD n. 19 (37 eyes)	HCs n. 17 (33 eyes)	p-value[*] (PD vs iRBD)	p-value[*] (iRBD vs HCs)	ANOVA p-value§
RNFL	13.4±1.9	15.4±1.8	17.8±2.2	<0.001	<0.001	<0.001
GCL	16.1±3.2	19.2±2.7	21.4±2.2	<0.001	<0.001	<0.001
IPL	21.4±2.9	22.5±2.8	24.2±2.1	0.10	0.005	<0.001
INL	20.7±5.5	23.2±3.9	28.2±4.5	0.02	<0.001	<0.001
OPL	28.5±6.3	27.1±3.4	31.2±4.8	0.21	<0.001	0.003
ONL	86.1±12.6	84.9±9.0	97.7±7.7	0.63	<0.001	<0.001
RPE	15.6±1.6	16.1±1.1	16.3±1.7	0.10	0.63	0.12
G ON	92.8±9.4	96.9±9.2	101.1±8.0	0.06	0.05	<0.001
SUP ON	112.8±16.5	114.8±10.0	120.6±9.6	0.52	0.02	0.03
TEMP ON	70.5±9.8	72.9±8.6	78.3±7.8	0.25	0.01	0.001
INF ON	120.2±15.0	119.2±9.2	119.4±9.2	0.73	0.94	0.92
NAS ON	73.0±10.2	79.5±8.5	80.9±8.2	0.003	0.48	<0.001

Legend: PD (Parkinson's disease), iRBD (Idiopathic Rapid eye movement sleep Behavior Disorder), HCs (healthy controls), RNFL: Retinal Nerve Fiber Layer, GCL: Ganglionic Cell Layer, IPL: Inner Plexiform Layer, INR: Inner Nuclear Layer, OPL: Outer Plexiform Layer, ONL: Outer Nuclear Layer, RPE: Retinal Pigment Epithelium, G ON: Global Optic Nerve, SUP ON: Superior sector - Optic Nerve, TEMP ON: Temporal sector - Optic Nerve, INF ON: Inferior sector - Optic Nerve, NAS ON: Nasal sector - Optic Nerve. * Using t-test, to compare two groups. §Using ANOVA-test, to compare three groups.

These findings have been confirmed by multivariate logistic regression analysis, adjusting by age, sex and hypertension (Supplementary table 3).

Supplementary table 3. Thickness of retinal layers in PD, iRBD and HCs group, adjusting by age, sex and hypertension.

	HCs versus iRBD				iRBD versus PD		
	AdjOR	95% CI	p-value		AdjOR	95% CI	p-value
RNFL	0.6	0.4-0.8	<0.001	RNFL	0.6	0.4-0.8	0.001
GCL	0.7	0.5-0.9	0.002	GCL	0.7	0.6-0.9	0.004
IPL	0.7	0.6-0.9	0.01	IPL	0.9	0.8-1.1	0.32
INL	0.7	0.6-0.9	<0.001	INL	0.9	0.8-1.0	0.08
OPL	0.7	0.6-0.9	0.002	OPL	-	-	-
ONL	0.8	0.7-0.9	<0.001	ONL	-	-	-
RPE	-	-	-	RPE	0.8	0.5-1.1	0.17
G ON	0.9	0.9-1.0	0.04	G ON	0.9	0.9-1.0	0.04
SUP ON	0.9	0.9-1.0	0.01	SUP ON	-	-	-
TEMP ON	0.9	0.9-1.0	0.02	TEMP ON	-	-	-
INF ON	-	-	-	INF ON	-	-	-
NAS ON	-	-	-	NAS ON	0.9	0.9-1.0	0.03

Legend: PD (Parkinson's disease), iRBD (Idiopathic Rapid eye movement sleep Behavior Disorder), HCs (healthy controls). Multivariate analysis, logistic regression, **adjusted** by age, sex and hypertension. RNFL: Retinal Nerve Fiber Layer, GCL: Ganglionic Cell Layer, IPL: Inner Plexiform Layer, INR: Inner Nuclear Layer, OPL: Outer Plexiform Layer, ONL: Outer Nuclear Layer, RPE: Retinal Pigment Epithelium, G ON: Global Optic Nerve, SUP ON: Superior sector - Optic Nerve, TEMP ON: Temporal sector - Optic Nerve, INF ON: Inferior sector - Optic Nerve, NAS ON: Nasal sector - Optic Nerve.

OCT analysis–Comparison of peripapillary RNFL among PD, iRBD and HCs

The overall optic disc and its superior and temporal sectors were thinner in iRBD and PD as compared to HCs (Table 2). These findings have been confirmed by multivariate logistic regression analysis, adjusting by age, sex and hypertension (Supplementary table 3). Moreover, comparing PD and iRBD subjects, the overall

optic disc and its nasal sector resulted to be significantly thinner in PD group, also after adjusting by age, sex and hypertension (Tables 2 and supplementary table 3).

OCT-A analysis–Comparison of microvascular density among PD, iRBD and HCs and association between retinal thickness and microvascular pathway

All PD patients and HCs underwent OCT-A evaluation while microvascular density was assessed in 14 out of 19 iRBD subjects (7 men, 50%; mean age 58.6 ± 13.4 years) One eye from PD patients, 1 eye from iRBD subjects and 1 eye from HCs were excluded because of the poor quality of OCT-A.

Comparing iRBD subjects with PD patients and HCs, a higher microvascular retinal density was found in iRBD group along the whole DCP, including its superior, inferior and parafoveal areas (Table 3). In iRBD, DCP resulted to be markedly increased with respect to superficial capillary density, as revealed by the ratio between superficial and deep capillary vascularization (Supplementary table 4). Indeed, considering each retinal sector, the ratio between superficial and deep capillary density resulted to be significantly lower in iRBD subjects as compared to both PD and HCs. Comparing iRBD and PD, this finding has been confirmed also by multivariate logistic regression analysis, adjusting by age, sex and hypertension (Supplementary table 4).

When conducting the correlation analysis, we did not find any significant correlations between retinal layers thickness and retinal microvascular pathway among iRBD subjects and HC subjects.

Table 3. Microvascular density pathway among PD, iRBD and HCs, assessed by OCT-A.

	PD n.21 (n. 41 eyes)	iRBD n.14 (n. 27 eyes)	HCs n.17 (n. 33 eyes)	p-value* (PD vs RBD)	p-value* (RBD vs HCs)	ANOVA p-value§
Foveal thickness	254.3±19.2	250.6±28.8	259.9±16.8	0.52	0.12	0.21
SCP whole	44.6±4.4	43.0±4.6	43.9±3.8	0.15	0.43	0.30
SCP superior	44.3±4.6	43.0±4.6	43.5±4.6	0.24	0.66	0.44
SCP inferior	44.9±4.3	43.0±4.6	43.7±3.8	0.09	0.52	0.18
SCP fovea	19.3±5.7	15.6±4.8	18.4±5.9	0.006	0.05	0.02
SCP parafovea	46.9±4.5	45.7±5.1	46.1±4.3	0.31	0.77	0.52
DCP whole	47.8±4.3	50.5±3.1	47.8±3.7	0.005	0.003	0.006
DCP superior	48.0±4.8	50.2±3.0	48.1±3.9	0.03	0.02	0.06
DCP inferior	47.6±4.3	50.9±3.2	47.5±3.8	0.001	0.001	0.001
DCP fovea	33.8±6.6	31.6±5.8	32.9±7.9	0.16	0.47	0.40
DCP parafovea	49.8±4.5	52.5±3.4	49.9±3.6	0.009	0.01	0.01

Legend: SCP: Superficial capillary plexus, DCP: deep capillary plexus.
* Using t-test, to compare two groups.
§Using ANOVA-test, to compare three groups.

Supplementary table 4. Ratio between superficial and deep capillary density in each retinal sector among PD, iRBD and HCs, assessed by OCT-A.

	PD n.21 (n. 41 eyes)	iRBD n.14 (n. 27 eyes)	HCs n.17 (n. 33 eyes)	p-value * (PD vs iRBD)	p-value § (PD vs iRBD)	p-value* (iRBD vs HCs)	p-value§ (iRBD vs HCs)
SCP/DCP whole	0.9±0.1	0.8±0.1	0.9±0.1	0.002	0.02	0.002	0.06
SCP/DCP superior	0.9±0.1	0.8±0.1	0.9±0.1	0.01	0.06	0.03	0.16
SCP/DCP inferior	0.9±0.1	0.8±0.1	0.9±0.1	<0.001	0.01	0.001	0.07
SCP/DCP fovea	0.6±01	0.5±0.1	0.5±0.1	0.004	0.02	0.008	0.17
SCP/DCP parafovea	0.9±0.1	0.9±0.1	0.9±0.1	0.01	0.02	0.02	0.24

Legend: SCP: Superficial capillary plexus, DCP: deep capillary plexus.
* Using t-test.
§ Multivariate analysis, logistic regression, **adjusted** by age, sex and hypertension.

Discussion

Our study demonstrated a thinning of different retinal layers (RNFL, GCL, IPL, INL, OPL and ONL) in both PD and iRBD patients with respect to healthy subjects. Interestingly, iRBD presented a retinal thickness that was intermediate between HCs and PD patients. This observation supports the hypothesis that retinal impairment is an early sign of neurodegeneration, occurring in the prodromal phase of PD, when only pre-motor symptoms are present, such as RBD.

Indeed, iRBD is considered one the most important marker of prodromal PD[4] and the presence of olfactory dysfunction, visual impairment, subtle motor signs, autonomic symptoms and abnormal dopaminergic imaging are supposed to be potential neurodegenerative biomarkers in iRBD, increasing the risk of conversion to PD[15,8,9,2].

Visual disturbances described in neurodegenerative diseases have been suggested to be related to dysfunction both in the visual cortex and in the retina[16]. OCT is a non-invasive and cheap technique used to investigated retina and optic disc. In several OCT studies, peripapillary RNFL resulted to be thinner in PD patients as compared to healthy subjects[17,6]. Moreover, many studies performing retinal segmentation analysis showed a thinning of RNFL, GCL, IPL, INL and OPL in PD patients[6] and it has been hypothesized that retinal thickness can be related to the visual impairment frequently reported by PD patients, also at early stage of disease[5]. Consistent with these data, we found a thinning of peripapillary RNFL

in PD patients as compared to healthy controls. Moreover, the thickness of each retinal layer resulted to be lower in PD patients as compared to HCs, except for RPE.

To the best of our knowledge, only two studies evaluated retinal thickness in iRBD. In particular, Yang and coll. reported that peripapillary RNFL is thinner in iRBD subjects with respect to controls[18]. Moreover, a thinning of peripapillary RNFL was found also in PD with RBD as compared to PD without RBD, suggesting RBD as a worsening factor[18]. However, the authors assessed the RNFL thickness in the peripapillary area of iRBD subjects but not the thickness of each retinal layer in the macular area. Conversely, Lee and coll. observed a thinning of ganglion cell complex (GCC) in iRBD as compared to HCs[10], with a value that laid between PD and controls.

In our study we evaluated the retinal thickness of iRBD patients using a retinal segmentation analysis by OCT. In agreement with data reported by Lee and coll.[10], we found a thinning of retinal layers in iRBD and in PD, as compared to healthy subjects, with values in iRBD group that are intermediate between HCs and PD patients. Thus, it could be hypothesized that retinal impairment occurs already in the prodromal phase of PD, representing an early sign of neurodegeneration. Then, retinal thinning could worsen with the progression of the neurodegeneration, reflecting a continuum of neuronal damage that begins already in iRBD patients and that continues up to the onset of PD, among which we found a macular RNFL

and GCL even thinner than in iRBD patients. The latter finding could be explained by the evidences that dopamine, physiologically released in the human retina by dopaminergic neurons[19], has a trophic role on retinal cells, including ganglionic cells. However, loss of dopaminergic cells and lower dopamine level have been described in PD patients' retina[20]. Moreover, dopaminergic neurons form synapses with ganglionic cells, providing not only a trophic support but also modulating visual pathway, whose output is represented by RNFL, axonal fibers of ganglionic cells[6].

Furthermore, phosphor-αSYN inclusions, histopathological hallmark of PD, have been found also outside of basal ganglia[21]. Phosphor-αSYN inclusions have been described also in the retina of PD patients and animal models, especially in RNFL, GCL and IPL[22]. Moreover, the phosphor-αSYN density in the retina has been described to significantly correlate with phosphor-αSYN density in the post-mortem brain of PD[23]. Thus, both dopamine depletion and abnormal alfa-synuclein (α-SYN) deposition in the retina could, at least partly, explain retinal impairment in PD. αSyn aggregates have been detected in submandibular gland nerve of iRBD patients[24] and, more interestingly, post-mortem studies revealed the presence of α-SYN deposition also in the brain of iRBD patients[25], indicating a neurodegenerative process at least in some iRBD subjects.

Nevertheless, the main novelty of our study was the assessment of microvascular retinal pattern in iRBD. Indeed, vascular degeneration has been well described in

PD patients but not in iRBD subjects. In particular, vascular impairment has been reported in PD subjects not only in brain regions associated to dopaminergic neuron degeneration (substantia nigra and brain stem nuclei), but also in regions not associated to dopaminergic degeneration, such as the middle frontal gyrus[26]. Moreover, in PD subjects a higher rate of string vessels has been reported as compared to healthy controls[27]. Conversely, to the best of our knowledge, this is the first study that evaluated retinal vascularization in a group of iRBD patients. Using OCT-A, we found that deep capillary density was remarkably higher than superficial capillary density in iRBD. Indeed, considering each retinal sector, the ratio between superficial and deep capillary density resulted to be significantly lower in iRBD subjects as compared to both PD and HCs.

We have not a clear explanation for such findings. Among possible factors, vascular remodeling due to an aSYN-induced inflammation might be supposed[22,28,29]. Indeed, it is known that abnormal protein deposition could activate microglial cells[30], leading to inflammation[31] and subsequent vascular changes, such as vasodilatation and neoangiogenesis[28]. Increased microglial activation has been reported in the retina of genetic rodent models of PD[22,30] and higher levels of pro-inflammatory factors have been described in post-mortem brains and cerebrospinal fluid of PD patients[32,33]. Interestingly, post-mortem studies revealed the presence of α-SYN deposition also in the brain of iRBD patients[24,25] and, recently, microglial activation has been observed also in the

substantia nigra of iRBD[34]. No data are available on the presence of α-SYN aggregates in the human retina of iRBD. Nevertheless, since retina is an extension of the brain, it could not be entirely excluded that inflammatory response occurring in the brain could also involve the retina[30].

As we previously said, we have not a clear explanation for our findings. Considering that RBD is the strongest prodromal feature of PD[4] and that inflammation leads to neurodegeneration[29,30], it could be speculated that RBD is a clinical-physiopathological "intermediate" condition between HC and PD, in which inflammation is more prominent than neurodegeneration, leading to a more prominent vasodilatation in DCP with respect to PD, where, conversely, neurodegeneration becomes more marked than inflammation. Nevertheless, this is just a speculation and further studies are needed to validate our hypothesis.

Several limits of our study should be taken into consideration in interpreting results. In particular, one important limit is related to the small size of our sample, because of which we cannot exclude that such findings might be due, at least partly, to chance. Moreover, not all iRBD subjects underwent an OCT-A evaluation. Thus, further studies with larger population are needed to verify our findings.

The lack of polysomnographic confirmation for some iRBD patents should be also taken into consideration. Diagnosis of RBD was, in fact, confirmed by VPSG recording only in nine subjects.

Nevertheless, considering VPSG as "gold standard", the RBD1Q has been reported to have high values of sensitivity and specificity, respectively 93.8% and 87.2%, with a positive predictive value (PPV) of 87.9% and a negative predictive value (NPV) of 93.4%[35]. In addition, patients whose RBD1Q was positive were extensively evaluated by a neurologist expert on sleep disorders in order to exclude other causes of secondary RBD before that pRBD diagnosis was made. Moreover, after comparing the thickness of each retinal layer between dRBD and pRBD, no significant differences were found. Nevertheless, the lack of polysomnographic confirmation for some iRBD patents should be taken into account for the interpretation of data.

In conclusion, retina resulted to be thinner in iRBD as compared to HCs, with a microvascular pattern different from both PD and HCs. All these findings point out the possible role of retina as a biomarker of neurodegeneration in iRBD and the opportunity to use non-invasive tools to select and monitor people at risk to evolve into neurodegenerative diseases. OCT and OCT-A might be some of these tools, certainly never alone and always along with more specific and largely accepted instruments. Extreme attention has been focused on iRBD, because it represents a "window of opportunity" in which experimental neuroprotective drugs could be tested, in order to act in the prodromal phases, before the occurrence of symptomatic and irreversible damage[9].

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5.0 General discussion

We have demonstrated that global prevalence of iRBD is less then 1% in the general population, when strict diagnostic criteria using VPSG are applied. Prevalence rates are higher (~5%) when broader diagnostic criteria are applied (e.g. using questionnaires or clinical confirmation).

On this aspect, we have highlighted three limitations in actual epidemiological investigations on RBD. First, the heterogeneity in survey methodology can affect the prevalence estimates, as such a coordinated effort to develop common guidelines for epidemiologists, should represent a priority in the following years. Issues such as the best cost/effective methodology (three or two stage design) or the inclusion of patients with a "prodromal RBD" [42] should also be reviewed. Second, the low compliance of the suspected cases to undergo a VPSG in several population-based studies is a major limitation that impacts the reliability of the prevalence estimates. To increase participations, awareness campaigns directed towards GPs, medical students and general population should be launched to promote knowledge on the disease and its implications, in order to increase the detection of potential cases that might otherwise be dismissed as non-pathologic sleep behaviors. Also, the use of domiciliary VPSG devices should be encouraged in epidemiological studies, with the possibility of incorporating wearable devices in the diagnostic process of RBD. Third, the lack of studies addressing the prevalence of RBD on different ethnicities (especially populations from South

America, Northern Africa and Sub-Saharan Africa) limits the understanding of possible genetic or environmental determinants on the prevalence of the disease. Global grant programs that promote studies directed towards these population could represent a possible solution for this issue in the future.

The need for precise estimate of iRBD prevalence in the general population will be progressively more relevant in neurological research, since this could be one of the future main target of neuroprotective therapies for alpha-synucleinopathies [40]. Moreover, increasing evidence points toward the clinical difference in the severity of disease between population based and hospital based iRBD patients [43], implying that patients discovered through large population-based surveys might represent an even earlier disease stage of alpha-synucleinopathy and thus those who might benefit more from neuroprotective strategies.

Once identified, the need for reliable biomarkers that could help differentiate iRBD patients at higher risk of phenoconversion to an alpha-synucleinopathy from patients that will not, will be a major challenge for researchers. Among all the possible instruments available [41], methodologies that are fast, easy to perform and non invasive should be preferred. In our third paper we have demonstrated that retinal analysis through OCT and angio-OCT is able to efficiently identify patients with RBD, when compared to healthy controls and PD patients. The OCT analysis is a fast and non-invasive possible biomarker, whose actual limitation is the use

confined to large hospitals and the technical expertise needed to correctly interpret

the results. Both of these limitations, however, could be easily overcome, if needed

for future trials.

6.0 Conclusions

Studying the prevalence of RBD is a challenging matter, with prevalence rates

highly dependent on the diagnostic methodology applied. In order to improve

research on this topic, a global effort towards standardization is required. At the

same time, studies implementing new biomarkers of phenoconversion are needed,

replicating the results obtained with the use of retinal thickness, possibly in a

longitudinal fashion, and promoting research on equally accessible and reliable

instruments.

7.0 General references

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8.0 Statements

1. "He was thrusting his sword in all directions, speaking out loud as if he were actually fighting a giant. And the strange thing was that he did not have his eyes open, because he was asleep and dreaming that he was battling the giant… He had stabbed the wine skins so many times, believing that he was stabbing the giant, that the entire room was filled with wine" (Don Chisciotte – Miguel De Cervantes).

2. "Striving to better, oft we mar what's well" (King Lear – William Shakespeare).

3. "No practitioner can do his daily work with any competence without constantly observing for himself, constantly reasoning from his own observations. The work of the medical practitioner, high or low, is personal science, as that of no other worker is" (Gowers 1895).

4. "There seems though to be a major difference between the two (a neurologist and an epidemiologist; *ndr*), reflecting the MD:PhD dichotomy. The physician has to treat his patient now with the best information he has; the epidemiologist can become so involved with caveats and possible exceptions that the major conclusions may well be obfuscated." (Kurtzke 2013)

5. "RBD is an "experiment of nature" in which knowledge from the study of motor-behavioral dyscontrol during REM sleep, with dream enactment, has

cast a broad and powerful light on a multitude of central nervous system disturbances, their evolution, and their comorbidities." (Schenck 2019)

6. According to our results, the overall prevalence of PSG confirmed, definite RBD is 0.68%. However, when considering studies that did not use the PSG as confirmation, thus evaluating only pRBD, the pooled prevalence rate rose to 5.65%.

7. Our study is the first VPSG based study on the prevalence of isolated RBD in Italy. Prevalence of isolated dRBD in our study was 0.26% but reached 0.59% when provRBD were also considered.

8. Our study demonstrated a thinning of different retinal layers (RNFL, GCL, IPL, INL, OPL and ONL) in both PD and iRBD patients with respect to healthy subjects. Interestingly, iRBD presented a retinal thickness that was intermediate between HCs and PD patients. This observation supports the hypothesis that retinal impairment is an early sign of neurodegeneration, occurring in the prodromal phase of PD, when only pre-motor symptoms are present, such as RBD.

9. The need for precise estimate of iRBD prevalence in the general population will be progressively more relevant in neurological research, since this could be one of the future main targets of neuroprotective therapies for alpha-synucleinopathies

10. The need for reliable biomarkers that could help differentiate iRBD patients at higher risk of phenoconversion to an alpha-synucleinopathy from patients that will not, will be a major challenge for researchers. Among all the possible instruments available, methodologies that are fast, easy to perform and non invasive should be preferred.