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MULTIMODAL BIOMARKERS STUDIES IN THE CONTINUUM DEMENTIA WITH LEWY BODIES – ALZHEIMER´S DISEASE

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"**Cover image**, courtesy of a patient, represents the experienced pareidolias when looking at the floor tiles"

CERTIFICATE OF DIRECTION

Dr. Alberto Lleó Bisa, who obtained his PhD in Medicine in the Universitat de Barcelona, Head of the Memory Unit in the Neurology Department in Hospital de la Santa Creu i Sant Pau and aggregate professor in the Universitat Autònoma de Barcelona and Dr. Juan Fortea Ormaechea, who obtained his PhD in Medicine in the Universitat de Barcelona, specialist in Neurology at Hospital de Sant Pau and medical director of the Alzheimer – Down Syndrome Unit

Certify:

That the work "Multimodal biomarkers studies in the continuum Dementia with Lewy bodies – Alzheimer´s disease", presented by Estrella Morenas Rodriguez to qualify for Doctor in Neurosciences for the Universitat Autònoma de Barcelona has been done under our direction and meets all the requirements to be presented and defended in the presence of the corresponding Thesis Committee.

Alberto Lleó Bisa,
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A mi Abuela,
por ti, para ti y contigo, siempre

A mis pacientes,
por ellos, para ellos y con ellos, siempre

ABSTRACT

Dementia with Lewy bodies (DLB) is characterized by its heterogeneity. In this thesis, I aim to investigate this clinical heterogeneity through the analysis of the different clinical subtypes based both on the predominant presentation features at the prodromal phase of the disease and by the assessment of sleep disorders, focusing on REM sleep behaviour disorder (RBD). I also investigate the role of glial activity in DLB by measuring the levels in cerebrospinal fluid (CSF) of glial proteins YKL-40, soluble TREM2 (*s*TREM2) and progranulin and its relationship with Alzheimer's disease (AD) biomarkers.

Patients with a clinical diagnosis of probable DLB were consecutively recruited during the period of this thesis. To capture the predominant clinical onset, I calculated the relative duration of each core symptom respective to the total disease duration from onset to fulfilment of probable DLB criteria. A K-means clustering method based on the initial clinical presentation was applied. A subset of patients also underwent a specific assessment of sleep including sleep scales and video-polysomnography (V-PSG). For the study of glial markers, we also included patients with prodromal DLB (prodDLB), AD dementia, prodromal AD (prodAD), and cognitively normal volunteers (CN). In this thesis I report three different clinical subtypes in DLB resulted from the cluster analysis performed. The cognitive-predominant subtype was the main subgroup obtained and was featured by a cognitive onset and a longer prodromal phase. Patients in the neuropsychiatric-predominant subtype were older at disease onset and were characterised by the predominance of hallucinations during the early stage. Patients in the parkinsonism-predominant subtype showed a faster decline until a dementia stage and a shorter time from onset to presence of parkinsonism. Regarding the sleep disturbances, a wide range of alterations were found in the subset

of patients specifically evaluated. Abnormalities in sleep-wake architecture were especially frequent. RBD was diagnosed in 50% of the studied patients, in most cases preceding the onset of cognitive impairment. Mimics of RBD were hallucinatory-like behaviours, severe obstructive sleep apnea and prominent periodic limb movements in sleep. Occipital EEG frequency while awake negatively correlated with the rate of electromyographic activity in REM sleep. Concerning glial markers, YKL-40, sTREM2 and progranulin levels did not differ between DLB groups and CN. However, YKL-40 levels, but not sTREM2 or PGRN, were higher in DLB patients who had a CSF profile suggestive of AD copathology than those without. In DLB and prodDLB, only YKL-40 correlated with t-tau and p-tau.

To conclude, this thesis investigates the heterogeneity in DLB and reports three clinical subtypes with different clinical profiles and progression patterns defined by the predominant features during its prodromal phase as well as the variety and complexity of sleep disorders in DLB, highlighting the need of V-PSG to properly identify them. This thesis also emphasises that CSF glial markers sTREM2 and PGRN are not increased in DLB and that YKL-40 is only increased in DLB patients with an AD biomarker profile, suggesting that this increase is driven by AD copathology.

LIST OF ABBREVIATIONS

^{123}Iodine-MIBG:	Metaiodobenzylguanidine myocardial scintigraphy
Aβ:	Amyloid β
AD:	Alzheimer 's disease
ADNI:	Alzheimer Disease Neuroimaging Initiative
AHI:	apnea-hypopnea index
ALS:	Amyotrophic lateral sclerosis
APOE:	Apolipoprotein E
CAF:	Clinician Assessment of Fluctuations
CBD:	Corticobasal degeneration
CN:	Cognitively normal
CNS:	Central nervous system
CPAP:	continuous positive airway pressure
CT:	Computerized tomography
CSF:	Cerebrospinal fluid
DAT:	Dopamine Transporter
DaTScan:	Dopamine transporter SPECT
DLB:	Dementia with Lewy bodies
EEG:	Electroencephalography
EMG:	electromyography
EOG:	electrooculography
FDG-PET:	Fludeoxiglucose positron emission tomography
FDS:	flexor digitorum superficialis
FTD:	Frontotemporal dementia
FTLD:	Frontotemporal lobar degeneration
GWAS:	Genome wide association study
iRBD:	idiopatic RBD
LB:	Lewy bodies
LN:	Lewy neurites

MCI:	Mild cognitive impairment
MMSE:	Minimental state examination
MoCA:	Montreal cognitive assessment
MRI:	Magnetic resonance imaging
MTL:	Medial temporal lobe
MS:	Multiple sclerosis
MSA:	Multisystem atrophy
MSQ:	Mayo sleep questionnaire
o-α-synuclein:	Oligomeric α-synuclein
ODFAS:	One Day Fluctuation Assessment Scale
p-α-synuclein:	α-synuclein phosphorylated at Serine 129
PD:	Parkinson disease
PDD:	Parkinson disease dementia
PET:	Positron emission tomography
PGRN:	Progranulin protein
PLMS:	Periodic limb movements in sleep
ProdAD:	Prodromal AD
ProdDLB:	Prodromal DLB
PSP:	Progressive supranuclear palsy
RBD:	rapid-eye-movement sleep behavior disorder
REM:	Rapid eye movement
NREM:	non-rapid-eye-movement
SN:	Substantia nigra
sTREM2:	Soluble TREM2
TLR:	Toll like receptor
TMT:	Trail making test
TNF:	Tumoral necrosis factor
TREM2:	Triggering receptor expressed on myeloid cell 2
UPDRS:	Unified Parkinson disease rating scale
V-PSG:	Video-polysomnography

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No sois sólo el último párrafo de estas palabras.
Gracias.

OUTLINE

This thesis aims to study the clinical heterogeneity of DLB. To achieve this objective, I have focused on the study of the clinical subtypes within the DLB syndrome with particular emphasis on the predominant clinical features of early disease. I have also studied the etiologic heterogeneity of the frequent sleep disturbances studied clinically and neurophysiologically by V-PSG and its implications in the clinical management and diagnosis. Finally, I have measured the biochemical markers of glial activation in CSF that may help in the identification of underlying neuropathological factors that contribute to this clinical heterogeneity.

This work has led to the publication of three articles, which have been included in this thesis, corresponding to the three objectives of this thesis. They are presented in Chapters III, IV and V. I have also included 6 articles as annexes to the main body of this thesis due to its relevance for the general discussion and related to the theme of this thesis.

Chapter I provides a general introduction to DLB from a clinical and pathological perspective and highlights the challenges in the diagnosis of this entity. From a clinical point of view, I highlight the important clinical heterogeneity present in the disease and the lack of diagnostic criteria for its prodromal phase as well as the etiological variety of the sleep disturbances that can influence the diagnosis and clinical management in a clinical setting. From a pathological point of view, I emphasise the presence of a frequent AD copathology that can influence the clinical heterogeneity and the relevance of the accompaniment of a glial contribution to synucleinopathies and specifically to DLB.

Chapter II presents the hypothesis and objectives of this thesis.

Chapter III describes the existence of three clinical subtypes of DLB with different evolutions, based on the symptomatology that predominates in the clinical onset of DLB and that were obtained by cluster analysis (K-means). It responds to hypothesis and objective 1.

Chapter IV assesses hypothesis and objective 2, elucidating the clinical and etiological heterogeneity of sleep disturbances in DLB confirming the need of a V-PSG for the correct diagnosis and management of them that may have important implications in the diagnosis and management of DLB.

Chapter V answers hypothesis and objective 3 and studies the difference in the CSF levels of the glial markers YKL-40, sTREM2 and PGRN in DLB compared to AD and its prodromal phases and the influence that AD copathology, reflected by core AD biomarkers, has on glial activity markers in DLB.

Chapter VI contains a summary of the results of the thesis.

Chapter VII presents a general and integrated discussion of the main results and remarks the importance of recognising the prodromal phases of the DLB as well as the knowledge of the diverse phenotypes to allow a deeper understanding of this disease.

Finally, it includes a future directions section based on questions that arise from this thesis that need further investigation.

CONTENTS

1. CHAPTER I: INTRODUCTION

1.1. History: from the discovery of Lewy Bodies to the current diagnostic criteria for Dementia with Lewy bodies

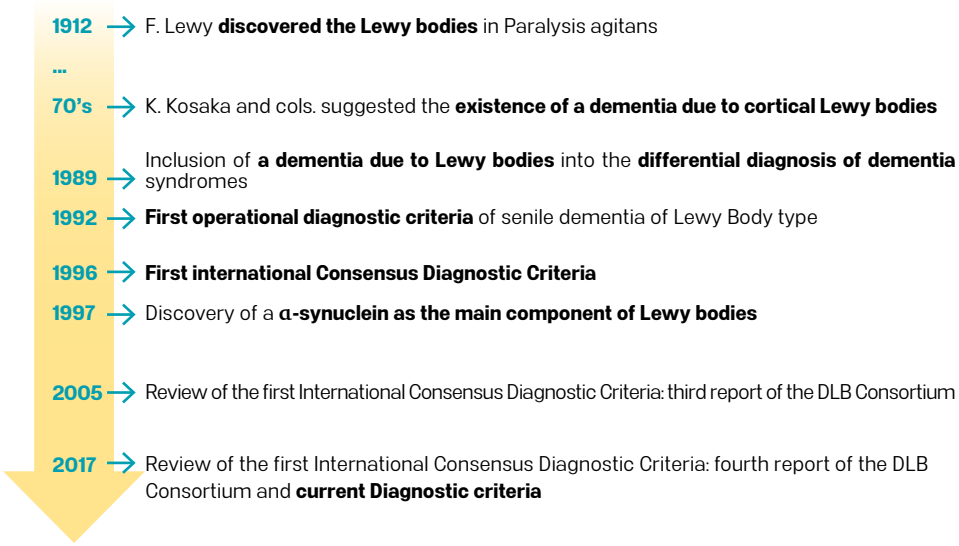
Lewy bodies were first described in 1912 by Friedrich Heinrich Lewy in his first publication on paralysis agitans, a disease that nowadays is known as Parkinson Disease (PD) (1).

However, at the time of their discovery, the relationship of Lewy bodies to cognitive impairment was totally neglected, and were believed to be associated with a purely motor disease (1). With the exception of some rare case reports (2,3), the concept of a dementia linked to Lewy bodies had to wait until the 70´s, when Kenji Kosaka and collaborators described several cases suffering progressive dementia and parkinsonism who presented with a neuropathology defined by cortical Lewy bodies (4,5). Subsequently, several similar series of patients with a clinical syndrome consistent of dementia and parkinsonism, and with a neuropathology characterized by cortical Lewy bodies with a variable degree of AD copathology were reported (6,7). These reports led to the inclusion of cortical Lewy bodies as a possible etiology for dementia, and the consideration of Dementia due to Lewy bodies within the differential diagnosis of dementia syndromes (7). Dementia with cortical Lewy bodies was thought to be a rare disease until 1992 when the first operational diagnostic criteria of senile dementia of Lewy Body type were issued (3).

In 1996, the first international consensus diagnostic criteria were published (8). Among the diverse terminology that had been used in the previous reports, the authors agreed on the denomination Dementia with Lewy bodies (DLB) to name the dementia syndrome characterized by a combination of

fluctuating cognition, recurrent visual hallucinations and parkinsonism (8). However, a number of controversies arose from these criteria, which mostly stemmed from the high prevalence of Alzheimer 's disease (AD) copathology. Of note, the concept of a variant of AD with Lewy bodies challenged these criteria depending on the requirement of both tangles and plaques to make a diagnosis of AD or if merely the presence of plaques alone was considered enough to diagnose AD. The low sensitivity of the criteria reported in subsequent studies was another important problem to solve and made evident that few cases were positively identified using these diagnostic criteria (9). It wasn´t until 1997 that Spillantini and colleagues discovered the main component of these pathological inclusions, α-synuclein (10) .
The first international consensus diagnostic criteria were revised in 2005. In this revision, experts emphasized the diagnostic importance of several previous supportive features, such as sleep disturbances, altered dopaminergic imaging, and the hypersensitivity to neuroleptics. Neuropathological diagnostic criteria were also reviewed, incorporating the probability of the clinical syndrome to be due to DLB or AD (11).

Figure 1: Timeline of main events and progression of diagnostic criteria of DLB



1.1.1. Disambiguation

As we have seen, the term DLB is relatively new and was consolidated in 1996 in the first consensus diagnostic criteria. Up to that point, the disease had had several denominations (8). Still today, maybe due to the low awareness of some aspects of the disease, and its overlap with PD and AD, a certain confusion in terms and concepts persists. In table 1 we summarize and disambiguate key DLB terminology.

Table 1. Definit*ion of the main terms related to DLB*

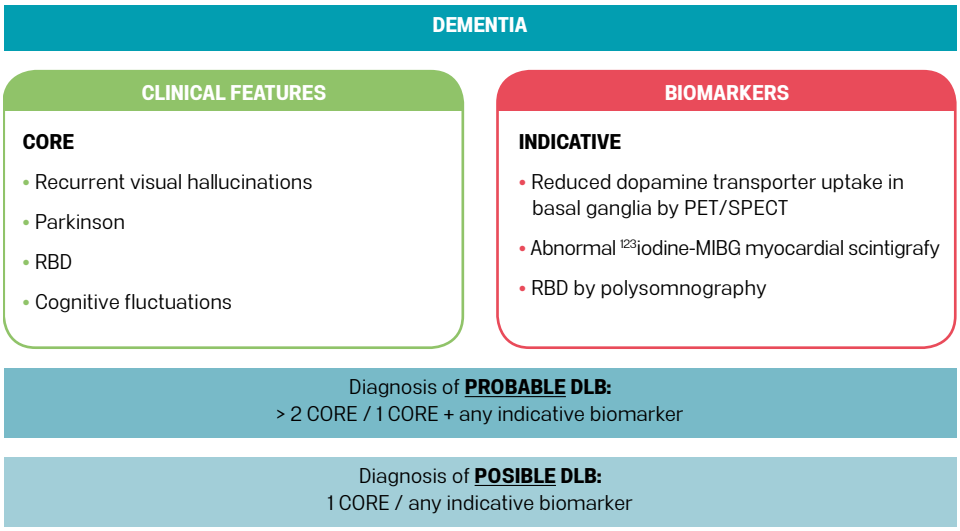
TERM	DEFINITION
DLB	Dementia with Lewy bodies as defined in the first section
Dementia in PD (PDD)	The dementia syndrome developed once a well-established PD is diagnosed. It is developed within the evolution of PD.
Lewy body dementia	Refers to DLB and PDD and indicates any type of dementia in which Lewy bodies are the main pathological finding.
Lewy body disease	The set of neurodegenerative diseases in which the main neuropathologic finding is Lewy bodies, namely, PD, PDD, DLB and multisystem Atrophy (MSA).
Variant of AD with Lewy bodies	AD with Lewy bodies restricted to the amygdala and absent from all other brain regions.

1.2. Dementia with Lewy Bodies from a clinical point of view

1.2.1. Definition and current diagnostic criteria

DLB represents 10 to 15% of all dementia cases, and is the second most common form of neurodegenerative dementia after Alzheimer´s disease (9). DLB is clinically defined by a syndrome consistent with progressive dementia associated with a variable combination of visual hallucinations, fluctuations, parkinsonism and REM sleep behavior disorder (RBD) (12,13).
Current clinical diagnostic criteria for DLB are summarized in Figure 2 (13)

Figure 2: Clinical diagnostic criteria for DLB according the fourth International Consensus



Dementia, understood as a progressive cognitive deterioration which interferes with the normal performance of an individual, is essential for the diagnosis of DLB (13). This cognitive decline in the context of DLB is featured by a relatively well conserved performance in memory tasks, and a more severe impairment of attention, executive and visual processing cognitive domains (14,15). Good scores in Memory and object naming with lower scores in processing speed and alternating attention are typical (14,15). Spatial and perceptual impairment are frequently detected in neuropsychological tests even early in the disease course (16,17).

Parkinsonism is the most common feature accompanying dementia in DLB. More than 85% of patients present spontaneous parkinsonian features in the disease course (13). In contrast to PD, only bradykinesia, rest tremor or rigidity in isolation is sufficient to diagnose parkinsonism in the context of DLB (13). In DLB patients, parkinsonism tends to be bilateral and symmetric and more bradykinetic than tremoric, with very frequent non-dopaminergic symptoms as frequent falls and hypomimia that can be often presenting symptoms (11,18).

Visual hallucinations are frequent in DLB, but despite having long been considered a hallmark of the disease, they are not a pathognomonic symptom and are not always present in DLB. When present, they are typically well formed, featuring people or animals, and very often well described by the patients. DLB patients have a variable capacity to critique the hallucinations and frequently have an emotional reaction to them despite being aware, in most cases, that the hallucinations are not real. They might also show a relative indifference to them more often than is the case for patients with other dementias (11,13).

Fluctuations are described as spontaneous alterations in cognition, attention and arousal including episodes of incoherent speech, behavioral variations and variable attention. However, even when they are frequent and typical, detection is difficult, and similar phenomenology can be present in other neurodegenerative dementias later in the disease course (13,19). There are several proposed scales for quantifying fluctuations (20).

Sleep disturbances, mainly RBD, are extremely frequent in DLB, influencing greatly the clinical picture and the quality of life in DLB patients. Consequently, the detection and treatment of sleep disturbances is crucial for the correct management of the disease (21–23). Other pathologies mimicking RBD are also very common and can make diagnosis and management difficult. We will discuss this aspect in more detail in the next section.

1.2.2. Biomarkers in Dementia with Lewy bodies

The main changes between the current clinical diagnostic criteria and the previous ones published in 2005 is a clear separation between clinical features and biomarkers, and an emphasis on the importance of RBD in the diagnosis of DLB (13).

Although there is no available biomarker to reflect Lewy body pathology, several other biomarkers are used in research studies and clinical practice. Moreover, in accordance with the new clinical diagnostic criteria for DLB, we can even state a diagnosis of DLB based on indicative biomarkers in the absence of any core clinical feature (13). The more clinical relevant biomarkers according to the last consensus diagnostic criteria are:

1.2.2.1. Indicative biomarkers.

Video-polysomnography (V-PSG). The demonstration of RBD or REM sleep without atonia is highly specific of an underlying synucleinopathy (22,23). As a result, the presence of a confirmative V-PSG alone is enough to make a diagnosis of DLB in a patient with dementia in the absence of any other core feature or biomarker (13). The following section and Chapter IV will address the relevance of this test in more detail.

Dopamine transporter (DAT) imaging. A decreased DAT uptake in basal ganglia, demonstrated by SPECT and PET imaging, can differentiate DLB from AD with a sensitivity and specificity of 78 and 90% respectively (24). It can also rule out the possibility of other overlapping syndromes, such as MSA, progressive supranuclear palsy (PSP) or corticobasal degeneration (CBD) (25,26). Nevertheless, normal DAT imaging is also possible as a result of symmetrical and balanced damage to the whole striatum rather than asymmetrical and predominant uptake reduction in the putamen (25,26). There are also DLB patients with a scarce brainstem involvement and low nigral neuronal loss that may result in a normal DAT imaging (27).

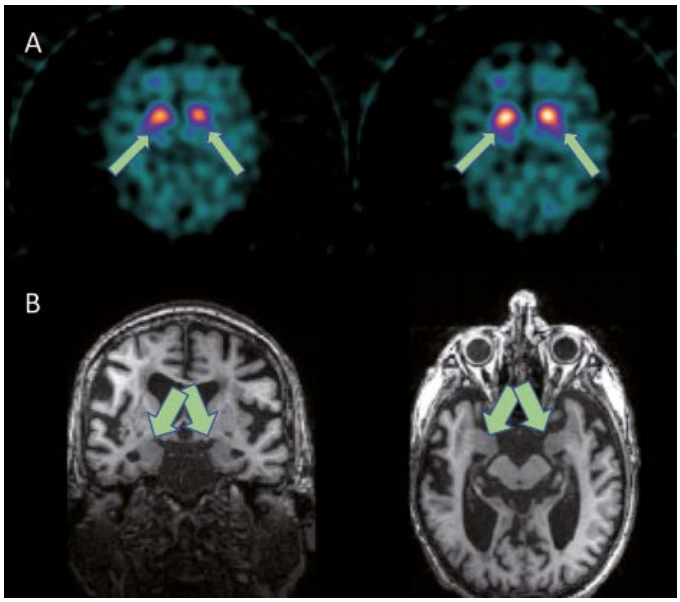


Figure 3. A: Abnormal DaTScan imaging showing bilateral and symmetric decreased DAT uptake in a DLB patient. B: MRI imaging showing the relative preservation of MTL structures in DLB.

Metaiodobenzylguanidine myiocardial scintigraphy (^{123}Iodine-MIBG). Reduced uptake of ^{123}Iodine-MIBG, a sign of damage to the postganglionic sympathetic cardiac innervations (13), is present in PD and DLB but not in AD, MSA, PSP or CBD. That being said, the high prevalence of other comorbidities in the elderly, such as diabetes mellitus or ischemic heart disease, as well as frequently prescribed medications that can alter the result of the test, thus reducing the clinical relevance of this biomarker (28).

1.2.2.2. Supportive biomarkers

Functional neuroimaging. Occipital hypometabolism demonstrated by 18F-Fluorodeoxiglucose positron emission tomography (FDG-PET) suggests a diagnosis of DLB rather than AD and correlates with visual cortex damage with a sensitivity and specificity of 70 and 74% respectively (29–31). The cingulate sign is described as the relative preservation of posterior or mid-cingulate cortex and is characteristic of DLB (32), correlating with lower neurofibrillary tangle burden but not with amyloid pathology (32,33).

Structural neuroimaging. The relative volume preservation of medial temporal lobe (MTL) evidenced by magnetic resonance imaging (MRI) or computerized tomography (CT) is suggestive of DLB when compared to AD. However, MTL atrophy is possible in DLB and may indicate higher AD copathology and faster cognitive decline (34).

Electroencephalography (EEG). A prominent posterior slow-wave EEG activity together with the possible presence of alpha/theta/delta activities in pseudoperiodic patterns is characteristic of DLB. It has a positive predictive value higher than 90% and correlates with the severity of clinical fluctuations. However, this EEG pattern may be rare in MCI stage (13).

1.2.2.3. Fluid biomarkers

Even when a diagnostic marker for DLB, either in blood or CSF, remains elusive, some biomarkers have been studied in these fluids in DLB. Concerning blood markers, an increase in the albumin CSF/serum ratio has been shown in DLB patients, but also in PDD and in stroke patients as a result of damage to the blood-brain barrier that is typical in these diseases (35). Higher plasma levels of annexin A5, a calcium and phospholipid binding protein, and transthyretin protein, which is involved in amyloid β (Aβ) clearance and maybe also involved in α-synuclein clearance, have been described in DLB, but also in AD (36,37). Blood markers for endothelial cell activation have also been reported to be altered in DLB and AD (38). Several studies have also failed to find specific biochemical markers for Lewy-related pathology in CSF. As the main component of Lewy related pathology, α-synuclein has been proposed as a potential biochemical marker for diagnosing DLB (39). The role of α-synuclein is not completely understood, but it may be involved in neurotransmitter release, vesicle trafficking, synaptic plasticity, brain lipid metabolism and membrane remodelling

(39,40). Previous studies have shown that the CSF levels of α-synuclein are decreased in PD and DLB and increased in AD and CJD (41,42). These findings suggest that this protein may not only be a marker of underlying synucleinopathy, but also a marker for synaptic damage and neurodegeneration (39,43). Thus, the diagnostic utility of CSF α-synuclein in DLB is limited (44). The levels of different species of α-synuclein have also been investigated in DLB and PD, mainly alpha synuclein phosphorylated at Serine 129 (p-α-synuclein) and oligomeric α-synuclein (o-α-synuclein) (42,45,46). An increase in CSF oligomeric α-synuclein has been described in DLB and PDD compared to AD (45). A recent study also showed a relative increase in its clinical utility when CSF total α-synuclein was combined with CSF total tau (44). Other combinations of several markers from diverse origins have been proposed to enhance the diagnostic accuracy of α-synuclein (47). Nevertheless, none of the described biomarkers are specific for DLB, and are usually common to AD and PD. Thus, the search for specific diagnostic markers for DLB that reflects the underlying pathophysiology is a research priority in the field of neurodegenerative dementias.

Core AD CSF biomarkers (Aβ40, Aβ42, t-tau and p-tau) have also been investigated in DLB. Different studies have correlated these AD markers with different clinical features in DLB, such as the presence of visual hallucinations or a faster cognitive decline (48). Additionally, AD core biomarkers can be useful in the clinical assessment of DLB, as CSF levels of tau and Aβ can detect those DLB patients with a higher burden of cortical Lewy body related pathology. Thus, these biomarkers can have important implications in prognosis and, when available, in the selection of individuals who could benefit from the administration of specific disease-modifying treatments that target both α-synuclein and Aβ (49).

1.3. Dementia with Lewy bodies from a neuropathologic perspective

1.3.1. Definition and current neuropathologic diagnostic criteria

Pathologically, DLB is defined by the presence of intraneuronal inclusions known as Lewy bodies (LB) and Lewy neurites (LN), which are also hallmarks of PD (11,13). In 1997, Spillantini et al discovered that the main component of these inclusions was α-synuclein (10). In DLB, α-synuclein is thought to spread inward from the olfactory bulb towards neocortical and lower brain areas, which contrasts the PD in which α-synuclein pathology is proposed to start in the brainstem and follow an ascendant spreading (50). The transmission α-synuclein from cell to cell of via endocytosis has been reported in cell models. In those models, dysfunctional quality control systems, particularly in lysosomes, promoted the formation of α-synuclein intracellular inclusions in the form of Lewy bodies or Lewy neurites (51).

The presence of Lewy bodies and neurites together with a compatible clinical syndrome during life are required for a pathologic confirmation of the DLB diagnosis (13). Alzheimer´s disease (AD) related pathology is a common comorbid pathology in most patients with a pathological DLB diagnosis (52). Considering this frequent AD copathology, the pathological diagnosis of DLB is based on the probability that the suggestive clinical syndrome is due to α-synuclein pathology, AD pathology or a combination of both. The pathological diagnostic criteria of DLB are summarized in **table 2** (13)

Table 2. Likelihood that the clinical syndrome is due to Lewy-related pathology. Based on the pathologic diagnosis scheme published by McKeith et al Neurology 2017.

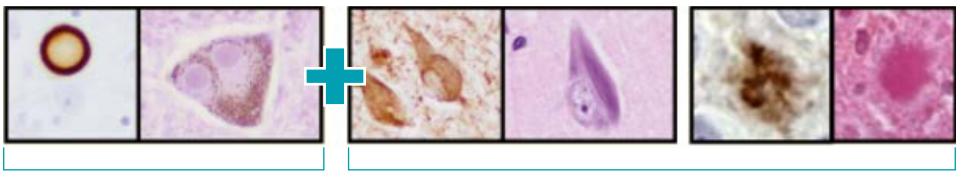
	AD PATHOLOGY		
Lewy-related pathology	NIA-AA none-low (Braak 0-II)	NIA-AA intermediate (Braak III-IV)	NIA-AA High (Braak V-VI)
Diffuse neocortical	High	High	Intermediate
Limbic (transitional)	High	Intermediate	Low
Brainstem-predominant	Low	Low	Low
Amygdala-predominant	Low	Low	Low
Olfactory bulb only	Low	Low	Low
Substantia Nigra neuronal loss should be assessed as none, mild, moderate and severe to classify cases according to the probability of having parkinsonism			

1.3.2. Common AD copathology

AD pathology in DLB tends to be more frequent in entorhinal cortex, amygdala and putamen (53). The percentage of patients with a diagnosis of DLB and AD copathology reported in the literature varies depending on whether the studies have been based on a pathological or clinical cohort and, in the latter, the method used to detect the suspected AD copathology. In neuropathological series, the percentage of DLB patients that also present sufficient AD copathology for a secondary pathologic diagnosis of AD is around a 50% (49,54). However, if we consider the presence of any degree of Alzheimer pathology, roughly 86% of pathologically confirmed DLB cases have AD copathology (55). By definition, neuropathological studies usually investigate end stage dementia. Therefore, these studies likely overestimate the contribution of AD copathology to the DLB phenotype. This overestimation may be more evident during the first years of the disease especially when considering the synergistic effect of AD and Lewy-related pathologies that will be more profoundly explained later in this section.

In vivo studies are essential to better understand the contribution of AD copathology to the clinical phenotype in DLB. Nowadays, Alzheimer's pathology can be studied in clinical cohorts using the core AD biomarkers in CSF and by amyloid imaging. Based on clinical cohorts in which AD copathology was investigated by amyloid imaging we know that concomitant Alzheimer's pathology depends on the age and the APOE genotype in DLB patients (56). APOE ε4 is a known genetic risk factor for AD (57), but also for α-synuclein pathology independently of AD copathology (58,59). Thus, AD copathology varies from 63% at 60 years old to 83% at 80 years old in APOE ε4 carriers DLB patients, and from 29% at 60 years old to 54% at 80 years old in noncarriers (56). In clinical a cohort studied by CSF biomarkers, the percentage of AD copathology suspected by a suggestive biomarker profile is 38% (48) But the variation can be high as it depends on the clinical cohort studied (older patients from a Geriatric Unit or younger patients from Sleep or Movement disorders clinics).

Figure 4. The AD copathology in DLB: Lewy bodies and Lewy neurites representing Lewy related pathology and neurofibrilary tangles and neuritic plaques representing AD copathology. Neuropathologic images courtesy of Marti Colom-Cadena.



In pathological series, the misdiagnosis of DLB, usually as AD, increases up to 50% (60), and is even higher when the cortical extension of α-synuclein is lower and the neuritic plaque burden is higher (61). Thus, the neuropathological diagnosis is crucial to properly detect DLB, the dementia most often misdiagnosed as other diseases (62).

1.3.3. Interaction between AD and Lewy pathologies

A synergistic effect between AD and Lewy pathologies has been proposed in DLB. Tau and α-synuclein pathology often colocalize in limbic areas, a brain area that has been proposed as an incubator for the spreading of misfolded proteins (63,64). Neuropathological studies have shown that the burden of tau pathology is one of the main factors related to the amount of cortical α-synuclein deposits (49,65). This is supported by other studies in which core AD biomarkers in CSF have been proposed as predictors of cortical α-synuclein (54). Cell culture studies have also investigated the synergistic effect between tau and alpha synuclein, finding that both misfolded proteins act together enhancing the cellular dysfunction and death (66). A shared seeding effect between α-synuclein, Aβ40 and Aβ42 has also been observed in vitro, with α-synuclein being attributed the increased seeding potential of the three (67). Thus, the interaction between these three misfolded proteins may contribute to increased seeding, cellular dysfunction and death and possibly to the trans-synaptic spread of both Lewy body and AD related pathologies (49).

1.4. Genetic Component of DLB

The genetic background of DLB is mostly based on common variants that confer a risk of suffering the disease rather than deterministic mutations causing the disease (68). However, the knowledge of the genetic architecture in DLB is more limited than in other neurodegenerative diseases due to the high rate of mis- and underdiagnosis, the lower investment in research into this disease, and because the disease and diagnosis have only relatively recently received much attention (69). In addition to this, DLB incidence is lower than AD or PD and the patients are typically older. Taken together, these factors have hindered the creation of big cohorts appropriate to genetic studies and the collection of biospecimens from a same family affected by the disease (69).

DLB shares some common genetic risk factors with AD and PD (68,69). The homozygosity of the most well-known risk factor for AD, *APOE* ε4, is also associated with an increased risk for DLB and disease severity (58,59,70,71). Its relationship with the concomitant AD pathology in DLB has been hypothesized (72), but this link with an increased risk for DLB, even in DLB with no or very low AD copathology, has been shown (70). *SNCA*, the gene encoding α-synuclein, has also been associated with increased risk for DLB and PD (69). Rare point mutations and locus multiplications (three or four copies) have been described causing familial DLB and other diseases as PD or, rarely, some cases of frontotemporal dementia (69,73,74). However, besides being a rare causative factor, *SNCA* has been also reported as a risk modulator for DLB with different association profiles at the *SNCA* locus (75). Decreased *SNCA* methylation has been linked to DLB (76). Heterozygous mutations in *GBA*, the gene responsible for Gaucher Disease and encoding the lysosomal enzyme beta-glucocerebrosidase, are related to a higher risk for DLB and PD (77–79). DLB cases linked to *GBA* variants are characterized by an early onset and faster evolution (78). Some studies have pointed out the relationship between the *MAPT* H1 haplotype and more severe α-synucleinopathy, as well as a link with clinical DLB (80–82). The *MAPT* variant p.Ala152Thr, related to AD and frontotemporal dementia, has been postulated to be associated with clinical DLB (83). Other shared genetic factors with AD, PD and frontotemporal dementia include mutations in *PSEN2*, *CHMP2B*, *PRKN* and *SQSTM1*, which were present in 4.4% of cases in a study of whole exome sequencing of 91 autopsy-confirmed DLB patients (71).

Besides the association of DLB with *APOE*, *SNCA* and *GBA* loci, which were confirmed by the recent GWAS (79,84), a new locus, *CNTN1*, has been associated with DLB (79). In addition, a recent study of genetic variability in DLB reported that copy number variation in certain loci may be related with a higher risk of DLB (85).

Overall, common variants have been proposed as the main contributors to the heritability in DLB. The estimation of the heritability based on these common variants increases from around a 30% in former studies to 59.9% more recently (86). Furthermore, these common variants have been proposed as an important substrate for the clinical heterogeneity featured in DLB independently of the variants contributing to the polygenic risk scores for PD or AD (79,86).

The DLB cohort studied in this thesis was also included in different multicentric and international genetic studies including two GWAS. The articles resulting from this research have been added as annexes.

1.5. Challenges in diagnosing DLB

1.5.1. Clinical heterogeneity

The order of appearance and severity of the core symptoms (parkinsonism, visual hallucinations, fluctuations and RBD) in DLB may vary from one patient to another, contributing to the heterogeneity in the phenotype. Moreover, not all DLB patients develop all the symptoms or show a similar ordering of presentation in the course of the disease. This results in different combinations of symptoms from which different phenotypes emerge. For example, a patient with dementia, parkinsonism and RBD and a patient with dementia, visual hallucinations and fluctuations, are both within the spectrum of DLB.

The clinical heterogeneity is broadly represented in the existing literature relating to the disease. The different core symptoms have a wide range of prevalence in the literature depending on the origin of the studied set/cohort of patients (clinical setting in which the patients have been evaluated). Some authors focused on the neuropsychiatric manifestations and

their studies frequently show a high prevalence of visual hallucinations and low of parkinsonism in their studies (3,87,88). Others focus on cognition or motor features picturing different phenotypes (18,23,89). Furthermore, depending on the presenting symptom, DLB patients may be referred to a memory, a movement disorder, a psychogeriatric or a sleep disorder unit where they will undergo different clinical assessments, which can further add to the clinical variability described in the DLB literature.

Compared to PD, where the study of the different phenotypes constitutes one of the main research priorities, clinical variability in DLB has received relatively little attention (90). In this field, recent studies have used a promising data-driven approach by clustering methods (18). Cluster analyses are a useful tool for deep phenotyping and have been previously used in other neurodegenerative diseases (91–93). One study using a hierarchical clustering method found two different distribution patterns of Lewy related pathology in demented patients with both, Lewy bodies and AD pathologies. The authors suggested that these different patterns may underlie the clinical heterogeneity of dementia due to Lewy bodies (93). Although different onsets and clinical subgroups have been proposed in DLB (94), clinical subgroups within the DLB spectrum have not been specifically studied by these unbiased data-driven tools.

The main differences in the clinical picture in DLB are more evident during the first years of the disease and the clinical features tend to be more homogeneous as the disease progresses reflecting a more global synaptic and neuronal degeneration. Therefore, in order to disentangle the high clinical heterogeneity observed in DLB it is crucial to focus in the initial presentation.

1.5.2. The biological basis of the clinical heterogeneity in DLB.

Previous studies have described different distribution patterns of Lewy body and AD pathologies in synucleinopathies that correlate with the phenotype (60,93). The different patterns and extent of those pathologies may affect the phenotypic variability in DLB as well as the clinical diagnosis during life (95).

Most pathological studies have observed a correlation between the amount of cortical α-synuclein aggregates with the duration of the disease (96,97). In fact, many cases of rapid progressive dementia clinically diagnosed as CJD had a high burden of Lewy body pathology together with concomitant AD (98). But, on the other hand, the amount of α-synuclein deposits does not seems to be related with other clinical outcomes during the disease probably because of the lack of neuronal death due to these inclusions(50,65).

In contrast, several studies have found a correlation between the cognitive impairment and tau deposition in DLB (49,65). AD copathology in DLB is related with a faster disease evolution, hallucinations and a worse performance in memory tests (48,99,100). It is also related with greater atrophy in medial temporal lobe, led by Aβ pathology and total atrophy and posterior grey matter atrophy that were related to total tau values (101). On the other hand, DLB patients with lower AD copathology, specifically, lower neurofibrillary and neuritic plaque burden, are more prone to have RBD as an early manifestation in the disease (102). Additionally, tau deposition patterns in DLB and AD are different, possibly contributing to the different phenotypes: in AD there is a higher burden of tau pathology than in DLB in all cortical areas, but while in AD there is a greater proportion of tau pathology in frontal neocortex, in DLB the proportion of tau pathology is bigger in temporal neocortex (65).

More recently, some authors have also stressed the importance of other features of α-synuclein pathology besides the amount and distribution of Lewy bodies and neurites in the phenotypical heterogeneity of DLB as well as in PD and MSA (60). Different experimental studies have demonstrated the existence of different strains of α-synuclein that can support this typical clinical heterogeneity among the synucleinopathies (103,104). In addition, elevated levels of insoluble p-α-synuclein have been found in the brain of patients with DLB but not in brains of PD or AD patients where the soluble p-α-synuclein and soluble o-α-synuclein is also higher than in controls, suggesting different changes in α-synuclein across the evolution of the different neurodegenerative diseases (105).

1.5.3. Prodromal DLB

Previous and current consensus diagnostic criteria of DLB require the presence of dementia. As such, the criteria neglect the existence of a prodromal phase of the disease. There are currently no established diagnostic criteria for the prodromal phase of DLB, which hinders the study of this stage as it makes selection of patients difficult, which in turn compromises the agreement and harmonization between researchers and centers (50). This lack of diagnostic criteria may be one of the reasons why literature on prodromal DLB is scarce, and restricted to retrospective studies or focused only on patients with RBD (94,106). Both instances may derive from biases as the features of DLB patients with or without RBD may vary (107).

Nevertheless, the study of the prodromal phase of DLB is crucial for several reasons. On the one hand, for a better understanding of the physiopathology of the disease and its progression. On the other, for the correct diagnosis of the patients from the beginning of their symptoms, which will help in determining whether to initiate treatment with appropriate disease-modifying drugs (when

available) and, from a social point of view, this will improve understanding and awareness of the patient, relatives and society to DLB (94).

Mild cognitive impairment (MCI) is the transitional stage between normal cognition and dementia in which cognitive decline does not affect the normal functioning in the patient's daily life (15,108). It has been profoundly studied in AD in contrast to DLB, where a great heterogeneity exists. Thus, the problem of the underdiagnosis of DLB becomes greater in the phase of MCI. The existence of diagnostic criteria and biomarkers in CSF for prodromal AD but not DLB means that clinicians have tended to misdiagnose DLB more frequently as AD. This etiologic label is more difficult to change later when other clinical features linked to DLB or the absence of AD suggestive biomarkers arise as the clinician must make this change understandable for patients and caregivers (94).

1.5.3.1. Clinical features of the prodromal phase of DLB

With the limitations commented above, most authors agree on the presence of a non-amnestic MCI as the main prodromal form in patients who are evolving to DLB (50,109–112). MCI-DLB is characterized by a predominantly dysexecutive cognitive profile, due to a dopaminergic dysfunction in fronto-striatal networks and relative preservation of hippocampus (109,112), with impairment in visual cognitive domains as well as social cognition deficits being common (50,110,111). The presence of multidomain amnestic MCI or even pure amnestic MCI, manifested by the impairment on free recall and visual recognition, is also present in the prodromal phase of DLB excluding the diagnosis of DLB in cases with amnestic MCI (110,111). DLB patients also present more often than AD patients with non-cognitive symptoms such as depression, visual hallucinations and RBD (50). In around one quarter of patients the presenting symptom is parkinsonism, with soft extrapyram-

idal signs being the best clinical predictor of evolution to DLB (50,113). In DLB, motor symptoms evolve slowly at the beginning, and faster near phenoconversion in contrast to PD, where progression of motor symptoms and signs followed a more linear progression. Regarding parkinsonian features, speech, voice, hypomimia, bradykinesia and reduced slim swim were the first manifestations, with rest tremor not as frequent at diagnosis and appearing only 2-1 years before phenoconversion, and with very low scores in the UPDRS-III (18). Delirium and transient alterations of consciousness are frequently reported in the early stage. On the other hand, fluctuations is the core feature more infrequently present at the prodromal stage of DLB, being more evident in later stages (50). When present, dream enactment is first referred 8.2 to 9 years before the diagnosis of DLB (18).

Some evaluations can facilitate DLB diagnosis in its prodromal stage. Within the neuropsychological evaluation, mistakes in the angles in the pentagon copy are highly suggestive of evolution to DLB in MCI patients (113). Regarding cognition, screening tests Montreal cognitive assessment (MoCA) scores starts to deviate from controls around 9 years before the diagnosis of DLB and Minimental state examination (MMSE), less sensitive in this pathology, deviates from the normal performance in a mean of 5 years prior to phenoconversion (18). At parkinsonism onset, semantic verbal fluency also predicted the development of DLB compared with PD (114). Verbal fluency is a useful test to monitor the cognitive evolution in DLB (115). When a more complete neuropsychological evaluation is available, tests assessing color vision can manifest an impairment more than 12 years prior the diagnosis of DLB (18), while trail making test -B (TMT-B) is impaired about six years before diagnosis (115). This points to a long prodromal phase in the disease that can be detected with the appropriate neuropsychological evaluation, especially when using tests directed towards assessing the executive and visuoperceptive domains.

1.5.3.2. Biomarkers in prodromal DLB

Structural neuroimaging studies have shown preservation of hippocampal volumes compared with prodromal AD that is of great help in the differential diagnosis between these two entities at the MCI stage (110,112). A bilateral grey matter atrophy in insulae and anterior cingulate cortex have been also described in prodromal phases of DLB (116,117). Functional neuroimaging studies consistently show specific hypoperfusion in temporal lobes in prodromal DLB (114,118) and hypoperfusion in the right frontal and parietal cortex together with the anterior insula as well as a hyperperfusion in left superior frontal gyrus are also described compared with healthy volunteers. When compared to prodromal AD, lower relative perfusion in prodromal DLB in the fusiform gyrus has been found (118). The presence of the cingulate sign may be useful for distinguishing prodromal DLB, although it is more evident in patients in the middle of the disease and decreases with the progression. When a posterior hypoperfusion is evident in prodromal DLB but the cingulate sign is absent it is indicative of a faster worsening and may point to a higher AD pathology (119). Dopaminergic imaging in prodromal DLB, has a lower sensitivity than in DLB, around a 50%, but it can be useful in the differential diagnosis in MCI as a positive dopamine transporter SPECT (DaTscan) points to an evolution to DLB with around 90% specificity (25,106). A reduced putamen to caudate ratio has been also described in prodromal DLB (114).

Most of the few existing studies on CSF biomarkers in prodromal DLB have focused on AD core biomarkers. Aβ40, Aβ42, t-tau and p-tau are less altered in the prodromal stage of DLB in comparison with the prodromal stage of AD, and are poor at differentiating prodromal DLB from controls (120). Nevertheless, the utility of AD core biomarkers in the clinical assessment of synucleinopathies, and specifically in the MCI stage of DLB has

been noted due to its link to a worse evolution and the possibility of detection those patients with an increased risk for higher cortical Lewy body related pathology (54).

1.5.4. Sleep Disorders in DLB

Sleep disturbances such as insomnia, hypersomnolence and abnormal sleep behaviors are frequent symptoms in DLB patients. In fact, some sleep disorders in subjects with dementia are indicative of DLB and rare in other conditions associated with cognitive impairment such as AD (121–123). That is the case of RBD. Its presence in patients with dementia supports the diagnosis of DLB (11,13). In fact, current diagnostic criteria of DLB consider RBD a core clinical feature of the disease, at the same level as fluctuations, visual hallucinations and parkinsonism (13)

RBD is a parasomnia consisting in the absence of atonia during the REM sleep and dream enactment. It can be present years before the onset of the first cognitive symptoms (21,124–127). RBD is manifested in nearly 76% of DLB patients during the course of the disease (125). Nevertheless, the identification of RBD in DLB is usually challenging because it can be mimicked by other sleep disorders that are also frequent in DLB as well as aging individuals. The main mimics are severe obstructive apnea, prominent periodic limb movements in sleep (PLMS), confusional awakenings or hallucinations (128–132). The absence of a reliable sleep informant is also frequent in DLB patients and complicates sleep assessment by the clinician. A useful tool capable of distinguishing mimics from RBD independent of the informant is V-PSG. Nevertheless, the current DLB diagnostic criteria do not require the performance of a PSG to confirm the diagnosis of RBD, which is included as a supportive biomarker (13).

Taken together and from a clinical point of view, sleep disorders cause additional disability, and influence negatively the quality of life of DLB patients. They can also worsen typical symptoms as daytime fluctuations or visual hallucinations, as well as cognition and lead to a more rapid deterioration. Their detection and appropriate treatment are crucial for the clinical management of DLB patients. Thus, a correct characterization of sleep disorders, such as RBD, in patients clinically diagnosed with DLB may help increase diagnostic accuracy and the implementation of adequate therapeutic strategies to improve patients' sleep and quality of life.

Few studies have addressed the sleep problems in DLB, and only very few of them have objectively evaluated sleep by nocturnal V-PSG. In those studies a higher prevalence of PLMS, shorter sleep latency, more frequent arousal episodes emerging from both, No-REM (NREM) and REM sleep and a variety of sleep disturbances besides RBD were found in DLB patients when compared with AD, PD or healthy volunteers (128,129,133–135). Nevertheless, few patients were included in most of these studies, and the polysomnographic findings lacked a detailed description. Therefore, a prospective study evaluating the presence of RBD and other sleep disturbances in DLB patients using subjective measures and a quantified V-PSG analysis is needed.

1.6. Neuroinflammation in DLB

Inflammation has been highlighted as an important player in the pathophysiology of neurodegenerative diseases such as AD or PD in epidemiological, pathological and genetic studies (136–138). Multiple studies have supported the importance of innate immunity, astroglia and microglia in neurodegeneration (139). These two cell types have very different functions in the central nervous system (CNS): microglia, the resident monocyt-

ic cells in the CNS, phagocyte cellular debris and protein aggregates, while astrocytes help in the neuronal and synaptic activity with neuroprotective role among other key functions (139).

The role of glial activation in tauopathies, and more broadly in AD, is being studied profoundly (140). Experimental in vivo and ex vivo studies have pointed to a reciprocal relationship in which tau pathology can affect microglial and astroglial function and, inversely, the chronic activation of glial cells can alter tau deposition and spreading with a negative impact in disease progression (136,141). In fact, loss-of-function variants of the gene for the triggering receptor expressed on myeloid cell 2 (TREM2), a microglial protein, increase the risk for AD, and constitute the second genetic risk factor for sporadic late-onset AD after the APOE ε4 allele (57,142). Moreover, ApoE is mainly secreted in the brain by glial cells and the ε4 allele has been found to have a decreased action in the suppression of inflammatory response (140,143). This strongly supports the importance of the immune response in the pathogenesis of AD.

The progranulin protein (PGRN) is mainly expressed by microglia, but also by neurons in the CNS (144,145). Genetic variants, such as the rs5848 polymorphism, that down-regulate expression of GRN, which encodes PGRN, have also been associated with an increased risk of AD (146–148). Of note these two proteins, TREM2 and PGRN, play an important role in the regulation of microglial activity (149,150). The suppression of the activity of TREM2 or PGRN produces opposite microglial activation profiles in mouse models (151). While a suppression of TREM2 activity locks microglia in a homeostatic state, PGRN suppression makes that microglia stays in a hyperactive disease-associated state (151). Thus, the study of these two proteins together is crucial to understand the role and changes of microglial activity along the neurodegenerative diseases.

Activated microglia can co-activate astroglial cells contributing to neuronal dysfunction and death, as astrocytes have an important function supporting neuronal health, changing its phenotype into a hyperactive state (152,153). These hyperactive astrocytes are upregulated in AD, and lose their capacity to promote neuronal survival, neuronal growth and synaptogenesis as well as having a reduced phagocytic function, being toxic for neurons (152).

Multiple lines of evidence support that glia are activated in synucleinopathies. Reactive microglial cells have been found in the vicinity of Lewy bodies, Lewy neurites and surrounding dying neurons in DLB (154,155). In particular, studies using 11C-PK11195 PET indicate the existence of activated microglia signal in the substantia nigra (SN) and putamen as well as several associative cortices in DLB patients (156). An increased expression of toll like receptor 2 (TLR2) in microglia in SN and hippocampus in incidental Lewy body pathology, has been found suggesting a role of microglia in the earliest phases of the disease (157). Thus, microglial activation in PD and DLB is believed to be involved in the initiation and progression of the disease including the secretion of pro-inflammatory cytokines and reactive oxygen species (158). In addition, synuclein released from neurons in PD and DLB can be endocytosed by astrocytes generating glial inclusions (159–161). These inclusions can induce changes in gene expression in astroglia, enhancing the inflammatory response and favouring neurodegeneration (161,162). A recent analysis of RNA levels in postmortem samples of DLB patients showed an increased expression of proteins that translate astrogliosis but not microgliosis (163). Processes of astroglia positive for α-TNF and inducible nitric oxide syntase have been detected near Lewy bodies (164). However, studies on the astrogliosis in PD brains have shown contradictory results, from a high astrogliosis to a low or absents astroglial pathology in brain regions affected by α-synuclein deposition, pointing to a high heterogeneity of astroglial activation and relationship with α-synuclein deposits within the disease(158).

In living people, it is possible to study the glial activation through the quantification of glial markers present in CSF. YKL-40 protein, also known as chitinase 3-like 1 protein, is a glycoprotein expressed by astrocytes near amyloid plaques in AD human brain (165,166). YKL-40 can be detected in CSF, its levels are increased in preclinical and prodromal AD, as well as in other neurodegenerative conditions, such as frontotemporal lobar degeneration (FTLD) or multiple sclerosis (MS) (167,168). Recent studies have shown an elevation in the CSF of a soluble fragment of TREM2 (sTREM2) in prodromal sporadic AD (169–171), as well as in preclinical and prodromal stages in autosomal dominant AD (172). In a similar way, PGRN has been reported to be increased even 10 years before symptom onset in autosomal dominant AD, while in sporadic AD its increase is observed in more advanced stages (173). Furthermore, higher CSF sTREM2 and PGRN levels during the early stages of AD are associated with a slower cognitive decline, lower hippocampal atrophy and less progression from MCI to a dementia stage (174).

Despite the number of studies linking glial activation in synucleinopathies, few have investigated glial activation markers in CSF in these disorders. Some studies have investigated the CSF levels of the astroglial YKL-40 protein in DLB, without differences in the levels between DLB or PD patients and controls (175–178). Only one study has investigated the CSF levels of PGRN in DLB and reported no difference with respect to healthy volunteers and other dementias (179). Until the work presented in this thesis, no studies had previously investigated sTREM2 in CSF in synucleinopathies.

Some studies have assessed the modulation of microglial activity as a potential treatment target in synucleinopathies. TLR4, expressed on microglia, has been implicated in α-synuclein clearance (180). The microglial

activation by an agonist of this receptor improved the movement as well as the α-synuclein cytosolic inclusions in a mouse model (181). Thus, although these glial markers have little diagnostic value themselves, they may be useful in the future to monitor disease-modifying treatments focused on the glial activation.

2. CHAPTER II: HYPOTHESIS AND OBJECTIVES

2.1. Hypothesis

1. There are different clinical subtypes in dementia with Lewy bodies (DLB) that can be better defined based on the clinical features in the prodromal stages of the disease.
2. Video-Polysomnography is essential to correctly diagnose the frequent and different sleep disorders associated with DLB.
3. The glial activation in dementia with Lewy bodies can be measured by inflammation-related biomarkers in CSF.
   - 3.1. The correlation between different glial biomarkers may depend on the underlying physiopathological processes.
   - 3.2. The glial-activation profile in DLB may be affected by AD con comitant pathology

2.2. Objectives

1. To define clinical subtypes using a data-driven approach based on the predominant symptoms during the prodromal phase of disease and to describe the features and evolution of the clinical subtypes resulted from the data-driven analysis.
2. To characterize the sleep disorders associated with DLB with a video-polysomnography study.
3. To determine the levels of certain microglial (sTREM2 and PGRN) and astroglial (YKL-40) proteins in CSF in dementia with Lewy bodies and its prodromal phase compared with AD, prodromal AD and healthy controls.
   - 3.1. To investigate the correlation between these 3 glial markers in dementia with Lewy bodies and AD.

3. CHAPTER III: CLINICAL SUBTYPES OF DEMENTIA WITH LEWY BODIES BASED ON THE INITIAL CLINICAL PRESENTATION.

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Running tittle: Clinical subtypes in dementia with Lewy bodies

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Key words: DLB, Lewy bodies, clinical subtypes, cluster analyses

Abstract

Objectives: To investigate the existence of clinical subtypes in dementia with Lewy bodies (DLB) based on the initial clinical presentation.

Methods: 81 patients with a clinical diagnosis of probable DLB were consecutively included. All patients underwent a neurological evaluation including a structured questionnaire about neuropsychiatric symptoms and sleep, an assessment of motor impairment (Unified Parkinson Disease Rating Scale subscale III) and a formal neuropsychological evaluation. Onset of core symptoms (hallucinations, parkinsonism and fluctuations) and dementia were systematically reviewed from medical records. We applied a K-means clustering method based on the initial clinical presentation. To capture the predominant clinical feature at the beginning of disease we calculated the relative duration of each core symptom respective to the total disease duration from onset to fulfilment of probable DLB criteria.

Results: Cluster analysis yielded three different groups. Patients in cluster I (cognitive-predominant, n=46) presented more frequently with cognitive symptoms (95.7%, n=44, $p<0.001$) and showed a longer duration from onset to DLB diagnosis ($p<0.001$) than the other clusters. Patients in cluster II (neuropsychiatric-predominant, n=22) were older at disease onset (78.1±5vs.73.6±6.1 and 73.6±4.2 in clusters I y III, respectively, both $p<0.01$), presented more frequently with psychotic symptoms (77.3%, n=17), and had a shorter duration until the onset of hallucinations ($p<0.001$). Patients in cluster III (parkinsonism-predominant, n=13) showed a shorter time from onset to presence of parkinsonism ($p<0.001$) and dementia (0.008).

Conclusions: three subtypes of clinical DLB can be defined when considering the differential initial presentations. The proposed subtypes have distinct clinical profiles and progression patterns.

Introduction

Dementia with Lewy bodies (DLB) is a heterogeneous condition from a clinical and pathological point of view (9,182). Pathologically, DLB is defined by the presence of inclusions constituted by aggregates of α-synuclein, known as Lewy bodies (LB) and Lewy neurites (LN), which are also hallmarks in Parkinson´s disease (PD). Alzheimer´s disease (AD)-related pathology is a common comorbid pathology in most patients with a pathological DLB diagnosis leading to a pathological heterogeneity (183). In addition, it has been shown that different distribution patterns of Lewy body and AD pathologies in synucleinopathies influence the clinical phenotype (93,182).

Clinically, DLB is characterized by a dementia associated with a variable combination of visual hallucinations, fluctuations, parkinsonism and REM sleep behaviour disorder (RBD) (9,11,13). However, not all patients develop all symptoms along the course of the disease and the order of appearance and severity may vary across patients leading to a variety of different phenotypes that may be related to the underlying physiopathology. For example, DLB patients with early RBD had lower neurofibrillary and neuritic plaque burden compared with those with late or absent RBD (184) while patients with hallucinations were more likely to have a cerebrospinal fluid (CSF) biomarker profile suggestive of AD copathology (48). Nevertheless, this variation in the clinical presentation of DLB and its underlying possible pathophysiology has been seldom investigated.

One of the current limitations in the diagnosis of DLB is the lack of established criteria for the prodromal phases of the disease (50). The previous and current consensus diagnostic criteria of DLB (11,13) require the presence of dementia. However, the main differences in the clinical picture in

DLB are more evident during the first years of the disease and the features tend to be more homogeneous as the disease progresses, likely reflecting a more global synaptic and neuronal degeneration. Therefore, in order to disentangle the high clinical heterogeneity observed in DLB is crucial to focus in the initial presentation.

Cluster analysis is a useful data-driven tool for deep phenotyping previously used in neurodegenerative diseases including PD (91–93,185,186). In DLB, there is just one study using a two-step clustering analysis to investigate the clustering of the core DLB symptoms in a group of patients with mild dementia (187). Nevertheless, the definition of clinical subgroups in DLB through data-driven tools has been barely investigated.
In this work, we used a cluster analysis to investigate clinical subtypes in DLB based on the initial clinical presentation and the predominant clinical features during the first years of the disease. Our hypothesis was that these measures could better reflect the different subtypes than measures obtained during later stages of the disease.

Materials and methods

Participants

We included 81 patients with a clinical diagnosis of DLB who were recruited consecutively in the Memory Unit of the Hospital de la Santa Creu i Sant Pau, between July 2013 and December 2015. Patients were referred by primary care physicians and evaluated in the Memory Unit by neurologists with expertise in dementia. Patients with suspected DLB were then referred to one of the investigators (EMR) for a specific evaluation. All patients were at a stage of mild dementia at inclusion, according to a consensus agreement between two experienced neurologists and one expert neuropsychologist based on core clinical criteria for dementia (188) and the score of the Clin-

ical Dementia Rating scale Sum of Boxes (CDR-SB) (189) and were diagnosed using criteria for probable DLB (11,13). The time from first symptom onset until the specific evaluation was 5 ± 3.2 years. All patients underwent a general neuropsychological assessment (190)and a specific neurological evaluation directed at detecting DLB signs and symptoms. The neurological evaluation included a structured questionnaire to interrogate about the features and onset of psychotic symptoms (hallucinations in different sensory modalities, sense of presence and passage hallucinations, illusions, misidentifications and delusions) and sleep, including clinical symptoms suggestive of RBD and its onset. The visit also included the assessment of motor impairment by means of the Unified Parkinson Disease Rating Scale subscale III (III-UPDRS). Fluctuations were quantitatively evaluated using the Clinician Assessment of Fluctuations (CAF) and the One Day Fluctuation Assessment Scale (ODFAS) questionnaires (20).

The following variables were collected in all patients in the structured questionnaire and the retrospective review of the medical records and during the first evaluation in the Memory Unit: first neurological or psychiatric symptom attributable to the underlying neurodegenerative process, date of onset of each core diagnostic feature (hallucinations, parkinsonism and fluctuations) and date of fulfilment of criteria for dementia (11). The date of disease onset was considered as the date of the first symptom reported. Data were coded as missing value when information was absent or inconsistent. We also reviewed the date in which each patient met probable DLB criteria considering all collected and reviewed variables and data.

To investigate the clinical heterogeneity during the first years of the disease we generated a paradigm to obtain a quantitative measure that could capture the length of each predominant core symptom before the diagnosis of DLB. Due to the difficulty for determining accurately the beginning of RBD

(lack of bedpartner and polysomnography in most cases) and fluctuations, we excluded these core symptoms in this quantification. The earliness of each selected core feature (hallucinations and parkinsonism) was estimated as the relative duration of each core symptom respective to the total disease duration from onset to fulfilment of probable DLB criteria as follows:
Time-to-dementia = the time from the date of first symptom to fulfilment of criteria for dementia.

- **TimeToDLBCriteria** = time from the date of first symptom to the fulfilment of probable DLB criteria.
- **Hallucinations duration** = TimeToDLBCriteria – (time from the date of first symptom to the beginning of hallucinations).
- **Parkinsonism duration** = TimeToDLBCriteria – (time from the date of first symptom to the beginning of motor symptoms/signs).

Then, to avoid the possible differences in the length of the clinical follow up, the following variables were calculated.
Earliness of hallucinations = (Hallucinations duration/TimeToDLBCriteria)*100
Earliness of parkinsonism = (Parkinsonism duration/TimeToDLBCriteria)*100
These calculated variables represent the percentage of the prodromal phase of the disease in which each single clinical feature was present.

Statistical analyses

We applied the K-means clustering method (91) to classify the subjects assuming three underlying subgroups based on their progression until the patient meet probable DLB criteria and the core symptoms of the disease: early presence of hallucinations, early presence of parkinsonism and none of those. Due to the difficulty for determining accurately the beginning of RBD (lack of bed partner and polysomnography in most cases) and fluctuations, we excluded these core symptoms in this analysis. The absence of

correlation between the selected variables in the cluster analysis (time to dementia, earliness of hallucinations and earliness of parkinsonism) was tested in order to avoid redundant information.

Then, we explored the descriptive features of each group and analysed their differences. Normality of the quantitative variables was explored by Shapiro-Wilk test. Variables with a non-normal distribution were analysed by means of a Kruskal-Wallis test, while those with a normal distribution were analysed by an ANOVA test. A chi-square test was used for categorical variables. Post-hoc analyses were performed also by means of a chi-square test for categorical variables, a Mann-Whitney U test in the case of non-normally distributed quantitative variables and a t-test in the case of quantitative variables with a normal distribution. Bonferroni correction for multiples comparisons was applied when more than two comparisons were made simultaneously.

Results

Demographics

The demographic and clinical data are described in table 1. The most common first symptom was cognitive impairment (67.9%) followed by psychosis (27.2%). Regarding the core symptoms and signs of DLB, 64.2% of patients had visual hallucinations at the time of inclusion, 96.3% had some degree of parkinsonism detected during the neurological examination, 84% referred cognitive fluctuations and 66.7% had clinical RBD.

Cluster solution and their clinical features

The main features of the three obtained clusters are specified in table 2.

Table 1. Demographics of the study participants.

	CLUSTER I (n=46)	CLUSTER II (n=22)	CLUSTER III (n=13)	TOTAL (n = 81)	P-VALUE
Gender % F (n)	56.5 (26)	68.2 (15)	53.8 (7)	59.3 (48)	0.67
Age at disease onset, mean (SD), y[a]	73.6 (6.1)	78.1 (5)	73.6 (4.2)	74.8 (5.9)	**0.007**
Age at evaluation, mean (SD), y[b]	79.5 (5.5)	82.2 (5)	77.1 (5.4)	79.8 (5.5)	**0.02**
Time to dementia, mean (SD), y[c]	4.2 (2.7)	3.1 (2.2)	2.3 (1.3)	3.6 (2.5)	**0.008**
Time to meet criteria of probable DLB [d]	5 (3)	3.1 (2.2)	2.4 (1.3)	4.5 (3)	**0.001**
Disease duration at evaluation, mean (SD) y[e]	5.9 (3.4)	4.1 (2.9)	3.6 (2.2)	5.0 (3.2)	**0.01**
Dementia duration at evaluation, mean (SD), y	1.8 (2)	1 (1.2)	1.3 (1.7)	1.5 (1.8)	0.495
INITIAL SYMPTOM[F]					
Cognitive (n)	95.7 (44)	22.7 (5)	46.2 (6)	62.5 (55)	**< 0.001**
Psychotic/behaviour (n)	4.3 (2)	77.3 (17)	23.1 (3)	27.2 (22)	
Motor (n)	-	-	30.8 (4)	4.9 (4)	

[a]Cluster I vs. II, p=0.003; Cluster II vs. III, p=0.009; Cluster I vs III, p=0.7
[b]Cluster II vs. Cluster III, p=0.008; Cluster II vs. Cluster I, p=0.058
[c]Time from the disease onset until the dementia stage. Cluster I vs. III: p=0.003; Cluster I vs. II: p=0.08
[d]Time from the disease onset until meet probable DLB criteria. Cluster I vs. Cluster III, p=0.001; Cluster I vs. Cluster II, p=0.009
[e]Time from the disease onset until the specific evaluation. Cluster I vs. Cluster II, p=0.02; Cluster I vs. Cluster III, p=0.015
[f]Number of patients with each initial symptom across diagnoses.

Table 2. Clinical features of resultant clusters

	CLUSTER I (n=46)	CLUSTER II (n=22)	CLUSTER III (n=13)	TOTAL (n= 81)	P-VALUE
CORE DIAGNOSTIC SYMPTOMS					
Parkinsonism % (n)	97.8 (45)	90.9 (20)	100 (13)	96.3 (78)	0.274
UPDRS score (part III), mean (SD)	22.2 (12.2)	21.7 (13.9)	26.1 (10.6)	22.7 (12.4)	0.384
Disease duration at onset of Pk, mean (SD), y[a]	4.6 (3)	2.3 (1.8)	0.3 (0.6)	3.3 (2.9)	**< 0.001**
Formed visual hallucinations % (n) [b]	54.3 (25)	100 (22)	2 38.5 (5)	64.2 (52)	**< 0.001**
Disease duration at onset of VH, mean (SD), y[c]	4.1 (2.9)	0.3 (0.6)	1.8 (1.3)	2.2 (2.8)	**< 0.001**
Fluctuations % (n)	84.8 (39)	90.9 (20)	69.2 (9)	84 (68)	0.234
ODFAS, mean (SD)	5.8 (4.5)	6 (3.8)	7.5 (5.1)	6.2 (4.4)	0.852
CAF, mean (SD)	6.5 (5.7)	8 (6.2)	7 (5.1)	7 (5.7)	0.659
Disease duration at onset of Fluctuations, mean (SD), y [d]	5.5 (3.1)	1.9 (2.5)	1.5 (1.4)	4 (3.3)	**< 0.001**
PSYCHOTIC/BEHAVIOURAL SYMPTOMS					
Delusions % (n)	41.3 (19)	63.6 (14)	46.2 (6)	48.1 (39)	0.223
Disease duration at onset of Del, mean (SD), y [e]	3.1 (2.5)	1 (1.4)	2.2 (1.9)	2.1 (2.2)	0.267
Misidentifications % (n)[f]	31.1 (14)	59.1 (13)	25 (3)	38 (30)	0.052
Disease duration at onset of Mis, mean (SD), y[g]	3.9 (2.7)	1.4 (1.6)	1.6 (0.8)	2.7 (2.5)	**0.046**
Sense of presence % (n)	19 (8)	31.8 (7)	23.1 (3)	25.3 (20)	0.351
Disease duration at onset of sense of presence, mean (SD), y	3 (3.5)	1.8 (1.9)	2.4 (1.3)	2.4 (2.6)	**0.076**
Passage hallucinations % (n)	26.2 (11)	31.8 (7)	7.7 (1)	30.4 (24)	0.322
Disease duration at onset of passage hallucinations, mean (SD), y[l]	5 (4)	2.1 (1.9)	1.2	3.7 (3.5)	**0.05**
SLEEP DISTURBANCES					
RBD % (n)	55.6 (30)	59.1 (13)	84.6 (11)	66.7 (54)	0.287
Disturbances in sleep cycle % (n)	47.8 (22)	59.1 (13)	23.1 (3)	46.9 (38)	0.117

[a]Cluster I vs. Cluster II and III and cluster II vs. Cluster III, p<0.001
[b]Cluster II vs. Cluster I and III, both, p<0.001
[c]Cluster I vs. Cluster II, p<0.001; cluster II vs. cluster III, p=0.001; Cluster I vs. Cluster III, p=0.09
[d]Cluster I vs. Cluster II and Cluster I vs. Cluster III, p<0.001
[e]Cluster I vs. Cluster II, p=0.008
[f]Cluster I vs. Cluster II, p=0.03
[g]Cluster I vs. Cluster II, p=0.03
[l]Cluster I vs. Cluster II, p=0.03
UPDRS: Unified Parkinson Disease Rating Scale; Pk: parkinsonism; VH: visual hallucinations; ODFAS: One Day Fluctuation Assessment Scale; CAF: Clinician Assessment of Fluctuations; Del: delusions; Mis: misidentifications; RBD: REM Sleep Behaviour Disorder

Cluster I or cognitive-predominant (n=46).

The first symptom of this cluster was mostly cognitive impairment with only two patients presenting with psychotic symptoms. Visual hallucinations (54.3%), parkinsonism (97.8%) and fluctuations (84.8%) appeared latter than in other clusters (all comparisons, $p<0.001$, table 2). Other psychotic and perceptive symptoms were less frequent, with a later onset when compared with cluster II. Two patients in this cluster referred isolated auditory hallucinations.

This cluster showed a longer time to dementia than cluster III ($p=0.003$) and a tendency for a longer evolution until the dementia stage than cluster II ($p=0.08$, table 2).

Cluster II or neuropsychiatric-predominant (n=22).

This group had an older age at disease onset compared with the other two clusters (both $p<0.01$, table 1). The most common first symptom in this cluster was psychosis (n=17, 77.3%). All patients had visual hallucinations and their mean onset was 0.3 ± 0.6 years after their first symptom, significantly earlier than patients in the other clusters ($p<0.001$).

Misidentifications were more frequent in this cluster when compared with cluster I ($p=0.03$). The frequency of sense of presence was lower than 35% in all clusters. However, the onset of this feature tended to be earlier in cluster II compared to the other clusters (table 2). The onset of passage hallucinations (26.2%) was earlier in cluster II compared to Cluster I ($p=0.03$). We did not find significant differences in the frequency of delusions between clusters (table2).

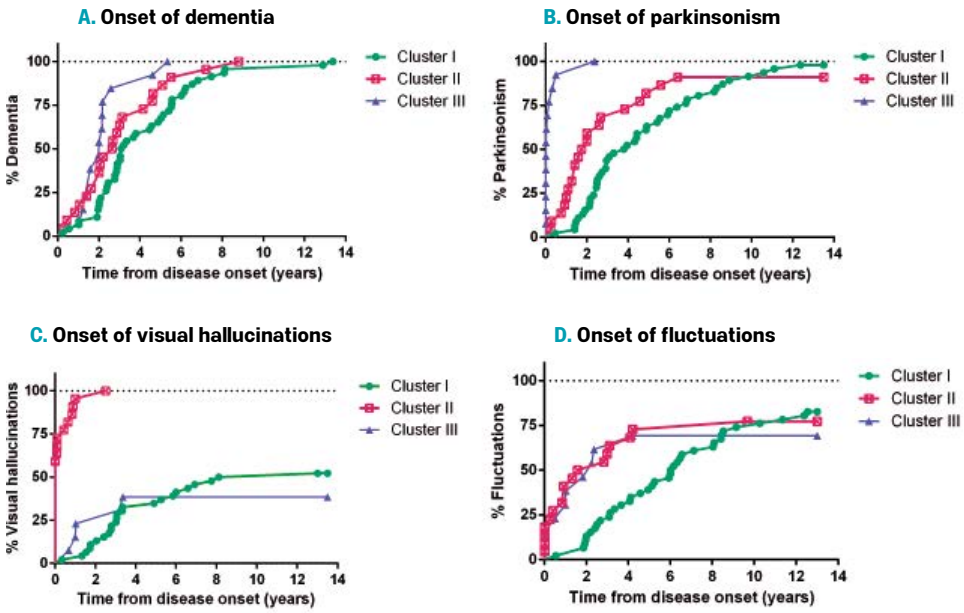
Cluster III or parkinsonism-predominant (n=13)

The mean age at onset of the disease in this cluster was 73.6 ± 4.2 years, younger than those patients in Cluster II ($p=0.009$) and comparable with

those in cluster I. The most common first symptom was cognitive impairment (six patients), followed by motor symptoms (four patients) and psychosis (three patients). All patients in cluster III had parkinsonism, with an onset of 0.3±0.6 years from the first symptom. Mean III-UPDRS score in cluster III was 26.1±10.6 without reaching statistical significance when comparing with the other two clusters (at inclusion, 11 patients out of 13 had a clinical suspicion of RBD). The time-to-dementia in this group was shorter compared to Cluster I (p=0.003).

Differential evolution from the disease onset of these three clusters is represented in **figure 1**.

Figure 1. Differential evolution from disease onset of the different resultant clusters, focused on core symptoms in DLB.



Neuropsychological assessment

Cognitive scores in neuropsychological examination at inclusion are summarized in **table 3**. Patients in cluster I had a worse performance in delayed free recall in the FCSRT when compared with patients included in cluster II ($p = 0.02$) and showed a trend for a worse performance when compared with cluster III ($p = 0.06$) in post-hoc analyses. There were no other differences between groups.

Table 3. Neuropsychological data of the resultant clusters

NEUROPSYCHOLOGICAL ASSESSMENT*	CLUSTER I (n=46)	CLUSTER II (n=22)	CLUSTER III (n=13)	TOTAL (n= 81)	P-VALUE
MMSE, mean (SD), n	22.6 (5.1), 46	24.5 (4.7), 20	25.3 (2.6), 13	23.5 (4.8), 79	0.109
Digit Span Forward-WAIS, mean** (SD), n	8.6 (2.5), 43	9.5 (2.3), 18	8.5 (4.1), 12	8.8 (3.1), 73	0.394
TMT-A, mean** (SD), n	4.7 (2.6), 43	4.2 (2.7), 18	3.8 (2.6)	4.4 (3.1)	0.775
FCSRT -Total recall-FCSRT, mean** (SD), n	4.2 (3.7), 43	5.9 (4.7), 18	5.7 (4.5), 12	4.8 (4.1), 73	0.269
FCSRT-Delayed free recall-FCSRT, mean** (SD), na	4.3 (3.5), 43	6.7 (3.5), 18	6.4 (3), 12	5.2 (3.6), 73	**0.07**
FCSRT-Delayed total recall, mean** (SD), n	5.3 (4.2), 43	7.4 (5), 18	7.6 (5.7), 12	6.2 (4.7), 73	0.225
Boston naming test, mean** (SD), n	7.3 (4.2),43	9.2 (3.5), 18	8.3 (4.4), 12	7.9 (4.1), 73	0.139
Rey-Osterrieth complex figure test- copy, mean** (SD), n	5.7 (2.7), 29	7.1 (2.7), 12	7 (4,5), 10	6.3 (3.2), 51	0.422
Number localization subtest-VOSP, mean** (SD), n	6.4 (3.9), 43	6.9 (4.4), 18	8.6 (4.8), 12	6.9 (4.1), 73	0.481
Digit Span Backward-WAIS, mean** (SD), n	9.6 (2.5), 43	9.9 (2.5), 18	10.4 (2.4), 12	9.8 (2.5), 73	0.291
TMT-B, mean** (SD), n	5.5 (2.6), 10	4.8 (2.4), 5	6.7 (4.7), 3	5.5 (2.8), 18	0.846

[a]Cluster I vs. Cluster II, p = 0.02; Cluster I vs. Cluster III, p=0.06.
*MMSE: Minimental State Examination, WAIS: Wechsler Adult Intelligence Scale, TMT-A: Trail Making Test A, FCSRT: Free and Cued Selective Reminding Test, VOSP: Visual Object and Space Perception battery, TMT-B: Trail Making Test B.
**Scalar scores according to age and educational level.

Discussion

In this study, we used for the first time a cluster analysis to characterize the different clinical subtypes of DLB based on the main clinical features during the prodromal phase of the disease. We obtained three clusters: a cognitive-predominant (cluster I), a neuropsychiatric-predominant (cluster II) and a parkinsonism-predominant (cluster III). These clusters differed in their clinical presentation, but also in the disease course. The cognitive-predominant cluster was characterized by a long prodromal phase in which cognitive symptoms, mainly memory complaints, predominated. Patients in this cluster had hallucinations and other psychotic symptoms less frequently than patients in other clusters and a later onset. Parkinsonian features also appeared later as compared to the other clusters. The neuropsychiatric-predominant cluster was characterized by an early onset of hallucinations and a higher frequency of psychotic symptoms as the presenting feature. Patients in this cluster had also a late disease onset compared to the other two. The parkinsonism-predominant cluster was featured by predominant motor symptoms during the first years of the disease, a faster progression from symptom onset to dementia compared to the cognitive-predominant cluster, and a low frequency of hallucinations compared to the psychotic-predominant cluster.

We have identified only one study using a data-driven approach to investigate the features of DLB in a cohort of patients with mild dementia (122). The authors found that the classical features of DLB aggregated together in their cluster solution supporting the diagnostic clinical criteria of DLB. However, clinical heterogeneity in DLB has not been specifically investigated by a clustering method. In contrast, previous studies have applied different clustering methods in PD avoiding subjective biases (93,191–193). Similar to our work, the studies performed in PD suggested subgroups differing in

their age-at-onset and rate of progression (191–193). In agreement with our data previous studies have described, that patients with earlier onset of parkinsonism are younger than those with earlier dementia or psychosis (194–196). It is remarkable that in some studies (191–193) the late-onset subgroup in PD was characterised by a faster clinical decline, differing from our late-onset cluster (cluster II, neuropsychiatric-predominant), defined by the presence of early hallucinations, but not a faster progression. Another described predictor for a faster progression in PD and DLB is the presence of concomitant AD pathology measured by CSF or amyloid-PET (48,195,197). It will be important to determine in future studies if those rapid evolution DLB clusters may have more frequency of concomitant AD. Selection of variables in cluster analysis is critical for the obtained solutions. In our study we focused on the clinical features present during the prodromal phase of the disease. These features may capture better the initial topography of neuronal dysfunction and loss (117,191,193). As the disease progresses, symptoms and signs tend to converge, reflecting more severe and widespread Lewy-body pathology and neurodegeneration resulting in a more homogeneous syndrome. Although criteria for prodromal DLB have been proposed (50,94,109), they are not completely accepted or validated. In fact, one of the challenges in defining criteria in the prodromal phase of DLB is the high heterogeneity observed during this disease stage (109,110). Our findings clearly support the notion that clinical heterogeneity is larger during the prodromal than the dementia stage. Our results showed that the neuropsychiatric and the parkinsonian-predominant clusters presented with clinical features that are characteristic of an underlying synucleinopathy. However, the cognitive-predominant cluster consisted of a syndrome of amnesic mild cognitive impairment (MCI) with a very slow progression. At the clinical level, this cluster partially overlaps with the typical amnesic MCI of AD (198). In fact, previous clinicopathological studies have suggested that Lewy body disorders should be considered in the

differential diagnosis of MCI as a subset of subjects convert to DLB during follow-up (110). The clinical heterogeneity in the prodromal phase of DLB and the potential overlap with a subset of patients with prodromal AD reinforces the need of developing specific CSF and/or imaging biomarkers for the initial stages of the disease.

It will be important to investigate how clinical heterogeneity in DLB relates to specific neuropathological profiles. Differences in the initial clinical syndrome could be the result of the different regional burden of α-synuclein pathology (64,93,183). A higher disruption of brainstem integrity could translate into more severe motor features, while a predominant cortical distribution may translate into predominant cognitive or perceptive disturbances (93,183). In addition, the common presence of coincident pathologies such as AD (93,199), could also influence the clinical manifestation of Lewy body disorders. We applied for the first time a K-means clustering method that yielded three well-defined clusters. The main strength of this study is the detailed characterization of the predominant clinical features during the prodromal phase by a replicable equation. This has been possible due to a structured chart review and questionnaire specifically oriented to detail the main clinical features during the prodromal phase of DLB. This data-driven approach allowed us to avoid multiple possible biases by defining particular time points.

The main limitations of the study are that part of the data was collected retrospectively, only a small subset of patients had imaging and/or CSF biomarkers, there was no validation in an independent clinical cohort and the absence of neuropathological findings to validate these clusters. In addition, in this study patients were recruited entirely in a memory unit, and this bias could underestimate the proportion of DLB patients with an initial motor presentation of the disease.

In summary, we propose three subtypes of DLB that differ in their clinical manifestations and progression patterns. It will be important to validate these subtypes in independent clinical cohorts with autopsy confirmation to investigate differences in biomarkers and neuropathological traits among the proposed clusters.

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Conflicts of interest

Authors declare no conflicts of interest

4. CHAPTER IV: CLINICAL AND VIDEO-POLYSOMNOGRAPHIC ANALYSIS OF REM SLEEP BEHAVIOUR AND OTHER SLEEP DISTURBANCES IN DEMENTIA WITH LEWY BODIES

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Title: Clinical and video-polysomnographic analysis of REM sleep behavior disorder and other sleep disturbances in dementia with Lewy bodies.

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Abstract

Objective: To study REM sleep behavior disorder (RBD) and other sleep disorders in dementia with Lewy bodies (DLB).

Methods: Consecutive patients with DLB and mild dementia severity were recruited irrespective of sleep complaints. Patients underwent clinical interview, assessment of sleep scales and video-polysomnography. RBD was diagnosed with video-polysomnography based on electromyographic and audiovisual analysis.

Results: Thirty-five patients (65.7% men; mean age 77.7 ± 6.1 years) were evaluated. Poor sleep quality (54.3%), hypersomnia (37.1%), snoring (60%), and abnormal nocturnal behaviors (77.1%) were reported. Sleep-wake architecture abnormalities occurred in 75% and consisted of occipital slowing on awake EEG (34.4%), absence of sleep spindles and K complexes (12.9%), slow frequency sleep spindles (12.9%), delta activity in REM sleep (19.2%) and REM sleep without atonia (44%). Three patients showed hallucinatory-like behaviors and ten abnormal behaviors during arousals mimicking RBD. RBD was diagnosed in 50% of those patients in whom sufficient REM sleep was attained. Of these, 72.7% were not aware of displaying dream-enacting behaviors and in 63.7% RBD preceded the onset of cognitive impairment. For RBD diagnosis, sensitivity of Mayo-Sleep-Questionnaire was 50%, specificity 66.7%, positive predictive value 83.3%, and negative predictive value 28%. False positive RBD cases according to clinical history had hallucinatory-like behaviors, severe obstructive sleep apnea and prominent periodic limb movements in sleep. Occipital EEG frequency while awake and rate of electromyographic activity in REM sleep were negatively correlated, suggesting a common subcortical origin.

Conclusion: In DLB, RBD and sleep-wake disorders are common, heterogeneous and complex, challenging their identification without performing V-PSG.

Keywords: Dementia with Lewy bodies, REM sleep behavior disorder, sleep disorders, video-polysomnography.

Statement of Significance

In DLB, neurodegeneration of the subcortical areas that modulate the sleep-wake cycle results in abnormal architecture (occipital EEG slowing while awake, lack of sleep spindles and K complexes and REM sleep without atonia) that can be identified using video-polysomnography. These abnormalities challenge the identification of wakefulness, sleep and its stages using standard scoring systems highlighting the need to design new scoring rules for patients with neurodegenerative diseases, such as DLB. In our study, some conditions were misdiagnosed as RBD when only the clinical history or the Mayo-Sleep-Questionnaire were used. This finding is relevant since current diagnostic criteria of DLB only require clinical history (and not video-polysomnography) to diagnose RBD, a parasomnia that is considered a core element of the disease.

Abbreviations

AHI: apnea-hypopnea index
CPAP: continuous positive airway pressure
DLB: dementia with Lewy bodies
EEG: electroencephalography
EMG: electromyography
EOG: electrooculography
FDS: flexor digitorum superficialis
MSQ: Mayo sleep questionnaire
NREM: non-rapid-eye-movement
PLMS: periodic limb movements in sleep

REM: rapid-eye-movement
RBD: rapid-eye-movement sleep behavior disorder
V-PSG: video-polysomnography

Introduction

Dementia with Lewy bodies (DLB) represents the second most common form of neurodegenerative dementia after Alzheimer disease.1,2 DLB is clinically characterized by dementia, fluctuating attention and alertness, recurrent visual hallucinations and parkinsonism.3,4 Sleep disturbances such as insomnia, hypersomnolence and abnormal sleep behaviors are also frequent in DLB patients. These disturbances contribute to a deterioration in quality of life and can be a therapeutic challenge.5-7 Some sleep disorders in subjects with dementia are indicative of DLB but not of other conditions associated with cognitive impairment such as Alzheimer disease.6-9 For instance, clinical and neuropathological studies have shown that REM sleep behavior disorder (RBD, a parasomnia manifested by dream-enacting behaviors and nightmares linked to increased muscular activity in REM sleep) is much more frequent in DLB than in other dementias.10-14

Current diagnostic criteria of DLB consider RBD a core clinical feature, but arguably do not require polysomnographic (PSG) confirmation (13). The clinical identification of RBD can be difficult because other conditions (e.g., severe obstructive apnea, prominent periodic limb movements in sleep, confusional awakenings, hallucinations) may mimic RBD symptomatology. Therefore, a definitive diagnosis of RBD requires PSG demonstration of REM sleep without atonia (128,129,131,132,135). Correct characterization of sleep disorders, such as RBD, in patients clinically diagnosed with DLB will improve diagnostic accuracy and lead to implementation of therapeutic strategies to improve patients' sleep and quality of life.

There are few studies addressing sleep problems in DLB and only a very few that have systematically evaluated sleep with objective tools such as nocturnal polysomnography with synchronized audiovisual recording (V-PSG) (21,121–123,126–130,133,134,202). In this study, we prospectively evaluated the presence of RBD and other sleep disturbances in a consecutive series of DLB patients using subjective measures and comprehensive V-PSG analysis.

Methods

Participants

Consecutive patients with clinical diagnosis of DLB (11) and mild dementia severity who underwent routine clinic visits between February 2014 and July 2016 at the Memory Units of the Hospital de la Santa Creu i Sant Pau and the Hospital Clínic de Barcelona, in Barcelona, Spain, were invited to participate in the current study irrespective of the presence and nature of sleep complaints. The diagnosis of DLB was based on the third report of the DLB consortium (where a history suggestive of RBD was not considered a core diagnostic feature) because our patients were recruited before the fourth report was published in 2017. Inclusion criteria included progressive dementia of mild severity with cognitive deficits and inability to perform complex tasks (level 4 of the Global Deterioration Scale) (203). Patients had to be on stable doses of medications for at least one month. DLB patients with moderate, moderately-severe and severe dementia (levels 5, 6 and 7 of the Global Deterioration Scale, respectively) were excluded. We also excluded subjects initially diagnosed in our institutions with isolated RBD by V-PSG who eventually were diagnosed with DLB.

Clinical assessments

Neurologists from the Memory Units (EMR, RSV, ALLA, ALLE) conducted a comprehensive neurological assessment that included clinical and demographic information, and current medications. Patients were referred to

the Multidisciplinary Sleep Unit of the Hospital Clínic de Barcelona where a neurologist with expertise in sleep disorders (AFA) invited them to 1) undergo a clinical interview covering aspects of sleep, 2) complete sleep questionnaires and, 3) undergo nocturnal V-PSG.

Sleep-wake assessments

Sleep-related symptomatology. This was examined with a comprehensive semi-structured sleep interview conducted by a sleep disorders expert (AFA) during which the presence of the bed partner or anyone who witnessed the patient's nocturnal sleep and sleep-wake pattern was encouraged to substantiate and complete the sleep history. The interview covered sleep habits and complaints such as sleep onset insomnia (difficulty in falling asleep within 30 minutes or more), sleep fragmentation (more than two awakenings lasting more than 15 minutes each), early awakening (waking up at least two hours earlier than desired), snoring, witnessed apneas, unpleasant dream recall and abnormal vocalizations and behaviors during the night. According to the sleep interview these sleep complaints were categorized as being present or absent in each participant. Overall sleep disturbances and nocturnal disability were also evaluated with the Pittsburgh Sleep Quality Index and a score >5 points was indicative of impairment of sleep quality (204). Daytime sleepiness was assessed by means of the Epworth Sleepiness Scale and a score >10 points was suggestive of excessive daytime sleepiness (205). To screen for a history suggestive of RBD, the Mayo Sleep Questionnaire (MSQ) was provided to the bed partner or another reliable informant (206). The MSQ was only administrated when the informant and the patient slept in the same room or when the informant had slept with the patient for years but was currently sleeping in another room due to the patient's sleep problems (206). Restless legs syndrome was diagnosed according to the International Restless Legs Syndrome Study Group criteria (207).

Video-polysomnography. PSG was performed with synchronized audio-visual recording (Coherence 7, Deltamed, Paris, France) and consisted in electroencephalography (EEG; F3, F4, C3, C4, O1, and O2, referred to the combined ears), electrooculography (EOG), electrocardiography, surface electromyogram (EMG) of the mentalis muscle, the right and left flexor digitorum superficialis (FDS) in the upper limbs and the right and left anterior tibialis in the lower limbs. Nasal cannula, nasal and oral thermistors, thoracic and abdominal strain gauges, and finger pulse oximeter were used to measure the respiratory variables. In patients who were using a continuous positive airway pressure (CPAP) mask before the start of this study, PSG was performed with CPAP and the air flow was measured with a pressure cannula connected to the mask, without thermistors.

Sleep and associated events were scored according to the American Academy of Sleep Medicine manual (208). Apnea was defined as a complete cessation of airflow for >10 seconds measured by the thermistor(208). Hypopnea was defined as ≥30% reduction in nasal pressure signal excursions from baseline and associated with ≥3% desaturation from pre-event baseline or with an arousal(208).The apnea-hypopnea index (AHI) was the number of apneas plus hypopneas per hour of sleep. Arousal was defined following the scoring rules of the American Sleep Disorders Association (209) The arousal index was the number of arousals per hour of sleep. Periodic limb movements during sleep (PLMS) in the legs were scored and the PLMS index was calculated as the number of leg movements per hour of sleep in a series of 4 or more movements with duration between 0.5 and 10 seconds and interval movement duration between 5 and 90 seconds (210).

EEG activity during wakefulness and sleep. The EEG activity during **wakefulness** was recorded at the beginning of the V-PSG while the patient was lying awake in bed. We visually evaluated 1) the dominant occipital back-

ground frequency (with the help of a power/frequency spectrum analyzer; Coherence 7.0, Deltamed-Paris, France), 2) the presence of abnormal slow activity, and 3) the reactivity to eye opening and closure. Reactivity of the EEG was considered abnormal when eye opening elicited only partial attenuation or no changes in the dominant occipital activity (211). Sleep stages and associated events were scored by two investigators (AFA, JS) according to standard criteria when possible (208), with specific considerations in patients in whom the standard criteria could not be applied because of abnormal sleep architecture (212,213). The following specific considerations were included:

a) Scoring **sleep onset** in patients with the persistence of the occipital alpha/theta activity that was seen in wakefulness when theta/delta activity in the frontal channels, slow eye movements in the EOG, and unequivocal behavioral manifestations of sleep (e.g., snoring) were recorded.

b) Scoring **stage N2** whenever K complexes or sleep spindles were present, even if the spindle frequency was <11 Hz (but was faster than the background occipital frequency seen in wakefulness) had typical sleep spindle duration (>0.5 seconds) and spatial distribution (maximal amplitude in central derivations) (208).

c) Scoring ***undifferentiated NREM sleep stage*** as those epochs with diffuse irregular delta-theta EEG activity of 0.5-4 Hz and 40-50 μV, that was clearly different from the dominant occipital background activity seen during wakefulness. This stage lacked vertex sharp waves, K complexes, sleep spindles and rapid eye movements. In some patients with *undifferentiated NREM sleep*, periods of delta-theta activity of 0.5-4 Hz with low to moderate amplitude (20-40 μV) were clearly distinguishable from other periods of delta activity of 0.5-1 Hz with higher amplitude (60-70 μV).

We termed these two types of periods light (amplitude 20-40 µV) and deep (amplitude 60-70 µV) *undifferentiated NREM sleep*, respectively.

d) Scoring **REM sleep** even in the presence of excessive EMG activity in the mentalis muscle channel and the occurrence of prominent intermittent delta activity in the EEG when other variables (e.g., rapid eye movements and typical behavioral manifestations of RBD) were highly indicative of this sleep stage.

Abnormal behavioral manifestations. We evaluated the motor and vocal manifestations detected in the audiovisual recording during wakefulness, the transition from wakefulness to sleep, the different sleep stages, and arousals, considering all the information obtained from the EEG, EOG, EMG and the audiovisual recording. The abnormal manifestations occurring upon arousals from sleep were classified according to the sleep stage that preceded the arousal, their apparent trigger, duration of the manifestations, the state of the eyes (open or closed), the type of observed movement or behavior, and its apparent emotional component during the event (quiet or agitated).

Diagnosis of RBD. Given the difficulties to obtain accurate information by clinical history about the occurrence and type of abnormal sleep behaviors in a number of patients (due to their cognitive problems or to the lack of a reliable informant) the presence of a chronic history of dream-enacting behaviors was not considered essential for the diagnosis of RBD in this study, although we evaluated its possible presence by means of the sleep expert interview and the MSQ. RBD was diagnosed in patients in whom V-PSG demonstrated excessive EMG activity in REM sleep linked to abnormal vocalizations, movements or behaviors detected in the synchronized audiovisual recording (214) For the measurement of EMG activity in REM sleep we followed the SINBAR method where a trained neurologist (AFA) visually quantified “any” (tonic, phasic or a combination of both) EMG ac-

tivity in the mentalis muscle and phasic EMG activity in the right and left FDS muscles (SINBAR montage) in 3-second mini epochs (215). Following this method, EMG activity in REM sleep was classified as excessive when 1) the rate of "any" EMG activity in the mentalis was ≥21.8% or 2) the rate of "any" EMG activity in the mentalis plus the phasic EMG activity in both FDS was ≥32%, measured in 3-second mini epochs (215). Because the EMG quantification of the mentalis plus the FDS is more sensitive to diagnose RBD than the mentalis alone, the former was used in all individuals except in one subject who due to artifacts in the upper limb EMG channels, EMG activity could not be measured in the FDS (215). RBD was also diagnosed in those cases in whom V-PSG demonstrated vigorous behaviors in REM sleep typical of RBD (e.g., repetitive prominent body jerking, punching, screaming) even though the quantified EMG activity in REM sleep was not excessive (216). In subjects with RBD, the intensity of the motor events and vocalizations seen in the video was classified as mild (e.g., body jerking, groaning), moderate (e.g., raising the arms, talking) or severe (e.g., punching, shouting) according to a system developed in our sleep center (217).

Patients with excessive EMG activity in REM sleep not associated with abnormal behaviors were categorized as having *isolated REM sleep without atonia and without RBD* (218).

We invited the patients to undergo a second V-PSG study when in the baseline study **1)** the total sleep time was less than 90 minutes, **2)** REM sleep was not recorded or lasted less than 10 minutes, **3)** RBD was not demonstrated when the clinical history was highly suggestive of this parasomnia and no other causes of dream-enacting were identified, and **4)** the need of CPAP titration to eliminate sleep breathing disorder in subjects with AHI >15.
The ethical committees at our institutions approved the study and all patients or their caregivers, when appropriate, gave written informed consent.

Statistical Analysis

Descriptive data are reported in mean, standard deviation, frequency and percentage. We analyzed differences between patients 1) who did and did not accept to participate in the study, 2) with and without RBD, and 3) with dominant occipital frequency during wakefulness faster or slower than 8 Hz. Comparisons were done using the Student t-test and Chi-square test. We performed Pearson correlations between the rate of EMG activity in REM sleep and the dominant EEG occipital frequency in wakefulness. For the diagnosis of RBD we calculated the sensitivity, specificity, positive predictive value and negative predictive value of the clinical interview by the sleep expert and of the MSQ. P values less than 0.05 were considered to be significant. All analyses were done with SPSS version 18.0 (Armonk, IBM Corp, NY, USA).

Results

Participants and clinical assessment

Eighty-two consecutive patients, 43 women and 39 men with a mean age of 78.8 ± 5.8 (range, 61-92) years were invited to participate and 35 (42.7%) accepted. There were more men in the group of patients who accepted to participate in the study compared with the group of patients who did not accept (65.7% vs. 34.0%, p=0.004). Other that the uneven gender distribution, there were no demographic or clinical differences between patients who accepted and did not accept to be enrolled (data not shown). Reasons for not participating were **1)** difficulty of the relatives and caregivers' to accompany the patients to the sleep visit and nocturnal V-PSG study, and **2)** patient refusal to undergo further assessments.

The 35 patients included in the study were 23 (65.7%) men and 12 (34.3%) women with a mean age of 77.7 ± 6.1 (range, 61-86) years and

a mean DLB duration of 0.9 ± 1.4 (range, 0.3-5) years. Thirty (85.7%) patients had parkinsonism, 24 (68.6%) fluctuations and 24 (68.6%) recurrent visual hallucinations. All but three patients were treated with central nervous system active medications including cholinesterase inhibitors in 26 (74.3%), antidepressants in 13 (37.1%), benzodiazepines in 12 (34.3%), dopaminergic agents in 11 (31.4%) and neuroleptics in seven (20.0%). Five patients were treated with clonazepam because their neurologists suspected RBD by clinical history (without having V-PSG confirmation). Two patients were using CPAP for obstructive sleep apnea that was diagnosed by PSG in other institutions.

Sleep wake assessments

Sleep-related symptomatology. All but one patient was accompanied by a family member or caregiver who helped to complete the sleep history, but only 17 (48.6%) had a reliable bed partner to describe the patients' nocturnal behavior. Six (17.1%) patients had sleep onset insomnia, eight (22.9%) sleep fragmentation, and six (17.1%) early awakenings. The mean Pittsburg Sleep Quality Index score was 6.8 ± 3.3 (range, 1-13) points, and 19 (54.3%) subjects had a score >5 points. The mean Epworth Sleepiness Scale score was 9.6 ± 5.3 (range, 1-24) points and 13 (37.1%) subjects had an Epworth Sleepiness Scale score >10 points. Twenty-one (60.0%) informants reported loud snoring and seven (20.0%) witnessed apneas during the patients' sleep. Six (17.1%) patients had restless legs syndrome. Ten (28.6%) patients experienced visual hallucinations only during the night or that were more vivid during darker periods of the day. Sixteen (45.7%) patients recalled unpleasant dreams. Abnormal behaviors at night were reported in 27 (77.1%) patients. Seven (20%) subjects had awakenings from nocturnal sleep with inappropriate responsiveness to the environment. Twenty-one (60%) had episodes of vocalizations and vigorous behaviors at night (e.g., talking, shouting, punching, falling or getting out

of bed). In 19 of these 21, the manifestations were suggestive of RBD according to the sleep expert. The abnormal behaviors resulted in injuries in eight (22.9%) individuals: ecchymosis in four, lacerations in three and both ecchymosis and lacerations in one subject.

Despite the high frequency of sleep complaints, only five (14.3%) patients sought medical help for sleep problems before the start of the current study.

Video-polysomnography (Figure 1, Table 1). Thirty-two of the 35 patients (91.4%) accepted to undergo V-PSG. Sleep was not attained in one patient who experienced hallucinations, agitation and aggressiveness during the V-PSG. In one patient only 26 minutes of stage N1 were recorded. In the remaining 30 patients more than 30 (range 98-432) minutes of sleep were recorded. Among these 30 individuals, REM sleep was not achieved in two patients. One subject achieved REM sleep for only 30 seconds and another for only 2.5 minutes. In 26 subjects REM sleep was recorded for more than three minutes with a mean of 46.5 ± 27.3 (range, 9.5-106.5) minutes.

Table 1. Sleep findings in the 31 patients who achieved sleep during video-polysomnography

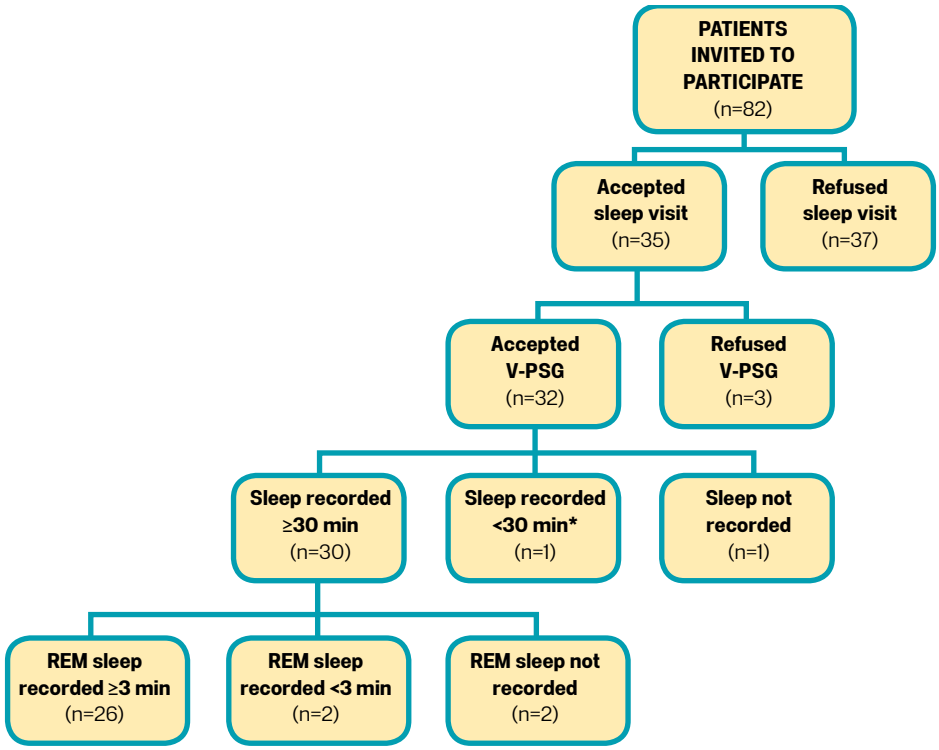
SLEEP PARAMETERS (n=31)		
Sleep efficiency, (%)		64.8 ± 20.3 (6.0-94.5)
Total sleep time, (min)		303.4 ± 93.4 (26.5-432.0)
Sleep onset latency, (min)		38.3 ± 50.9 (0.5-233.5)
Awakenings, (n)		7.7 ± 12.1 (0-59.5)
Arousal index (n)		26.2 ± 17.1 (2.2-71.2)
AHI, (n)		18.9 ± 16.3 (0-59.6)
CT90, (%)		11.2 ± 21.9 (0-90)
PLMSI, (n)		26.7 ± 35.9 (0-136)
NREM SLEEP PARAMETERS (n=31)*		
Conventional NREM sleep (n=26)	N1 (%)	19.4 ± 15.0
	N2 (%)	57.9 ± 14.4
	N3 (%)	12.5 ± 13.9
Sleep spindle frequency (Hz) (n=26)	9.5 Hz (n, %)	1(3.8)
	10 Hz (n, %)	2 (7.7)
	10.5 Hz (n, %)	1 (3.8)
	11 Hz (n, %)	4 (15.4)
	≥12 Hz (n, %)	18 (69.2)
Undifferentiated NREM sleep (n=4)		
Stable undifferentiated NREM sleep (n=1)(%)		91.6
Undifferentiated light and deep NREM sleep (n=3)	Light NREM sleep (%)	51.6 ± 17.8
	Deep NREM sleep (%)	26.2 ± 3.5
REM SLEEP PARAMETERS (n=28)**		
REM sleep latency, (min)		182.9 ± 116.9 (51.5-430.0)
REM sleep, (min)		43.2 ± 28.8 (0.5-106.5)
REM sleep, (%)		14.3 ± 8.4 (0.5-28.5)
Presence of sawtooth waves, n (%)		10(38.5)
Background EEG activity, n (%)	Alpha	4 (14.3)
	Theta	17 (60.7)
	Delta	5 (17.9)

SLEEP PARAMETERS (n=31)	
30-second epochs of REM sleep, (n)	80 ± 50.2 (11-215)
3-second mini epochs of REM sleep, (n)	813.8 ± 513.4 (132-2136)
Phasic and tonic EMG activity in the mentalis, (%) ***	24.1 ± 24.9 (0-100)
Phasic and tonic EMG activity in the mentalis plus phasic EMG activity in the FDS in REM sleep, (%) +	31.9 ± 27.7 (0-100)
Excessive muscular activity, n (%) ++	11 (44.0)
Abnormal movements and behaviors, n (%)	13 (50.0)
Excessive EMG activity plus abnormal movement and behaviors, n (%)	11 (42.3)

Values are expressed as mean ± standard deviation, number, frequency and range. AHI: Apnea-hypoapnea index; CT90: Percentage of sleep time with oxyhemoglobin saturation below 90%; PLMSI: Periodic leg movements in sleep index; NREM: Non rapid eye movements; EMG: Electromyography; FDS: Flexor digitorum superficialis.

*: One patient only achieved N1 and 21 patients did not achieve N3.
**: REM sleep was not recorded in three of the 31 patients that underwent v-PSG and sleep was recorded.
***: Percentage of phasic and tonic electromyographic activity in the mentalis muscle measured in mini epochs of 3-seconds in the patients who achieved REM sleep without artifacts in the mentalis muscle lead (n=26).
+: Percentage of phasic and tonic electromyographic activity in the mentalis muscle plus phasic electromyographic activity in the bilateral flexor digitorum superficialis measured in mini epochs of 3-seconds in the patients who achieved REM sleep without artifacts in the electromyographic leads (n=25).
++: ≥31.9% of combined phasic and tonic electromyographic activity in the mentalis muscle plus phasic electromyographic activity in bilateral flexor digitorum superficialis in 3-seconds mini epochs.

Figure 1. Flow chart describing the recruitment and video-polysomnographic findings of DLB patients.



Abbreviations: V-PSG: Video-polysomnography; *: REM sleep was not attained in this patient, only stage N1.

We offered repeat V-PSG to the eight patients in whom sleep was not recorded (n=1), REM sleep was not attained (n=4), or REM sleep was achieved for less than 10 minutes (n=3). Five of these eight patients were taking antidepressants. Of these eight patients, three accepted to undergo a second study, and REM sleep was achieved in two.

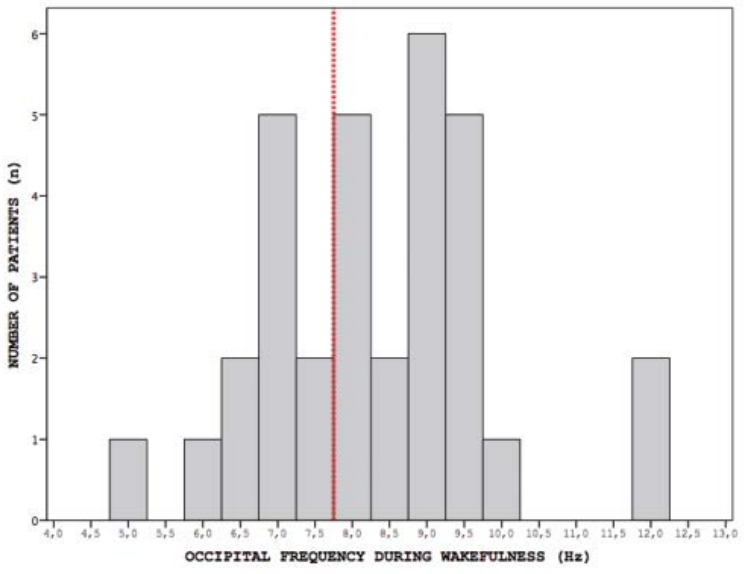
In the 31 patients in whom sleep was recorded during the V-PSG, sleep efficiency was 64.8 ± 20.3%. The mean AHI was 18.9 ± 16.3. A V-PSG study with CPAP titration was offered to the 14 (45.2%) subjects who had an index >15, but only four accepted. The mean PLMS index was 26.7 ±

35.9, and 14 (45.2%) patients had an index >15. In 10 (32.3%) patients PLMS were distributed during NREM sleep across the whole night and in two patients persisted during REM sleep. Periodic leg movements during wakefulness occurred in 10 (32.3%) patients.

EEG activity during wakefulness and sleep. Abnormal EEG activity during wakefulness and sleep occurred in 24 of the 32 (75.0%) patients who underwent V-PSG.

During wakefulness, 21 (65.6%) subjects had alpha rhythm of 8-12 Hz, and 11 (34.4%) had dominant occipital frequency in the theta range between 5 and 7.5 Hz (Figure 2). Four (12.5%) patients had diffuse continuous theta activity; with superimposed almost continuous delta activity in three, and with intermittent bursts of delta activity in one. Decreased reactivity to eye opening was present in 14 (43.7%) patients, the majority (71.4%) with occipital background frequencies <8 Hz.

Figure 2. Distribution of the frequency of the posterior background electroencephalographic activity during wakefulness.

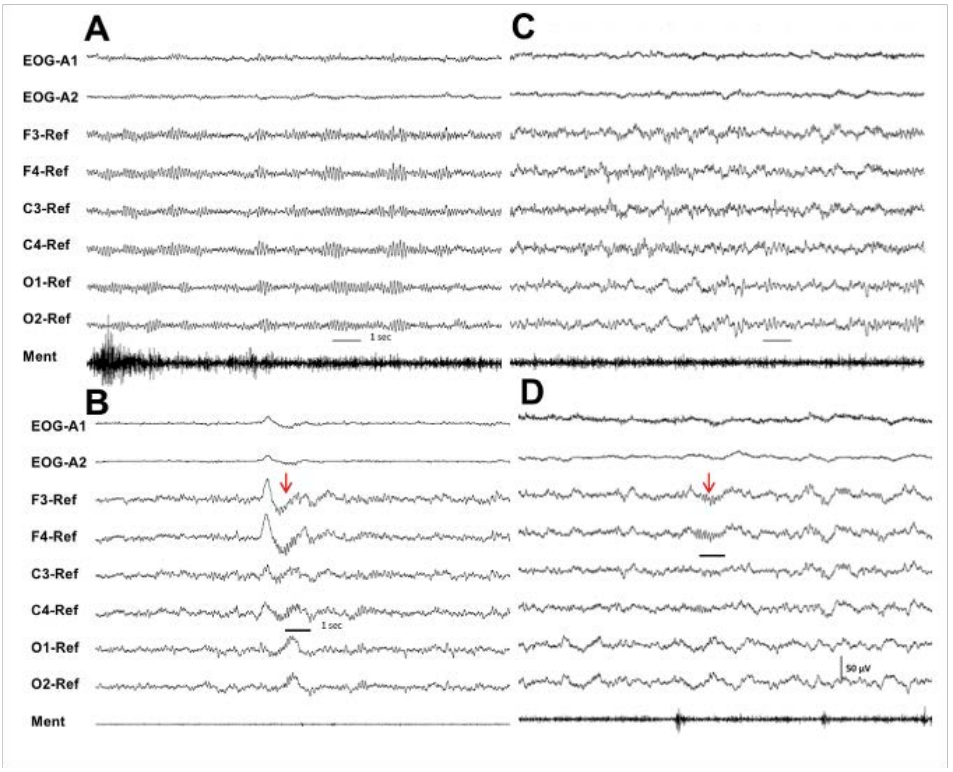


In the 31 patients who achieved NREM sleep, one (3.2%) patient only attained stage N1, while conventional NREM sleep was recorded in 26 (83.9%) and undifferentiated NREM sleep was recorded in four (12.9%). In NREM sleep, two patients had occasional sharp waves (one patient in stages N2 and N3, and the other only in stage N2). Neither of the two patients with sharp waves had experienced seizures during sleep or had a past medical history of epilepsy.

At sleep onset, eight patients showed persistence of the dominant occipital alpha/theta activity but it was at least 1 Hz slower than the occipital frequency recorded during wakefulness. Two patients (one with alpha rhythm and one with theta activity during wakefulness) had frontal intermittent rhythmic delta activity (FIRDA) at sleep onset. In four (12.9%) cases NREM sleep onset was characterized by continuous sustained EMG activity in the mentalis, and occasional irregular bursts of semi-rapid eye movements in the EOG (that is, conjugate eye movements with an initial deflection lasting >250 msec or <250 msec but with lower amplitude than those typically seen in REM sleep in the same patient). One of these four cases was treated with selective serotonin reuptake inhibitors.

Stage N2 was identified in 26 patients. The mean sleep spindle frequency was 12.2 ± 1.4 (range, 9.5-14) Hz. The sleep spindle frequency was slower than 11 Hz in four (15.5%) subjects and slower than 12 Hz in eight (30.8%) (Figure 3).

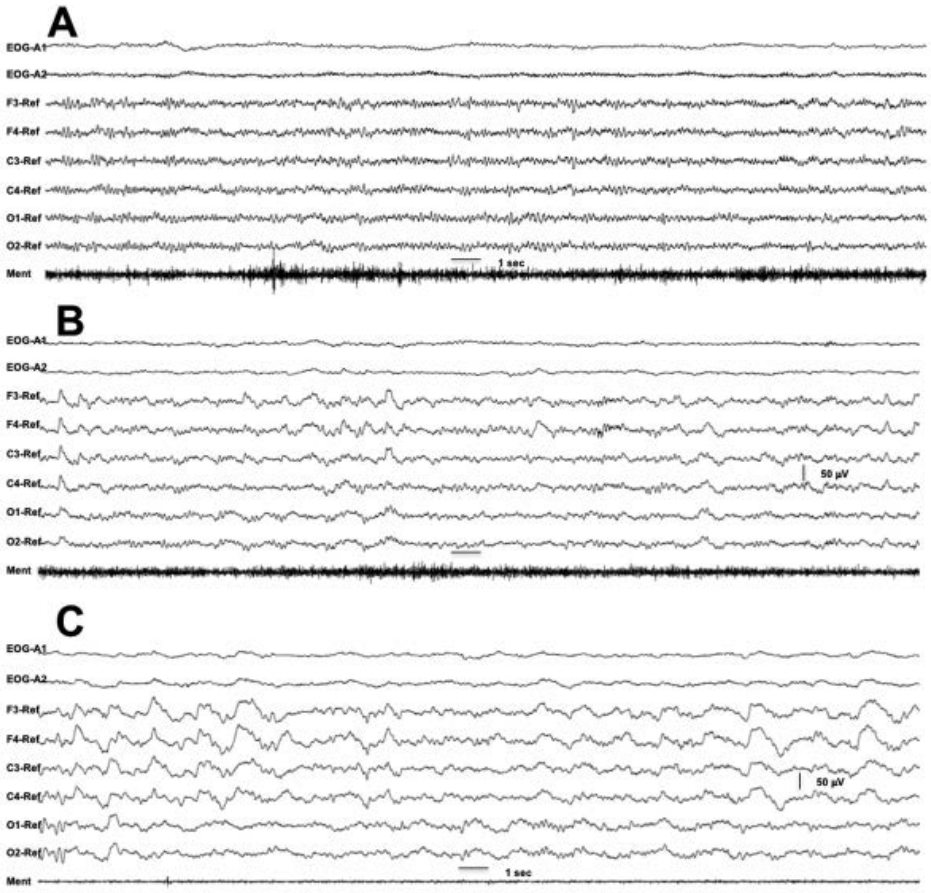
Figure 3. Polysomnographic features during wakefulness and stage N2 in two patients with normal and slow frequency sleep spindles. Patient 1. A: Quiet wakefulness with normal alpha rhythm (10 Hz); B: Stage N2 with normal sleep spindle frequency (13 Hz, arrow). Patient 2. C: Quiet wakefulness with diffuse theta activity (7 Hz); D: Stage N2 with slow sleep spindle frequency (10 Hz, arrow).



Abbreviations: EOG: electrooculogram; EEG: electroencephalogram referenced to both ears; Ment: electromyography of the mentalis muscle. Horizontal bar represents one second. Vertical bar represents 50 µV.

Undifferentiated NREM sleep was identified in four patients. Three had periods of both light and deep undifferentiated NREM sleep, while the remaining subject had no changes in the amplitude of the slow EEG activity during this stage (Figure 4).

Figure 4. Undifferentiated light and deep NREM sleep in a patient with slow background frequency awake. A: Quiet wakefulness with diffuse, posterior predominant, continuous theta activity. B: Undifferentiated light NREM sleep with diffuse, moderate amplitude irregular delta/theta activity in the absence of K complexes and sleep spindles. C: Undifferentiated deep NREM sleep with diffuse, higher amplitude and slower delta activity in the absence of sleep spindles.

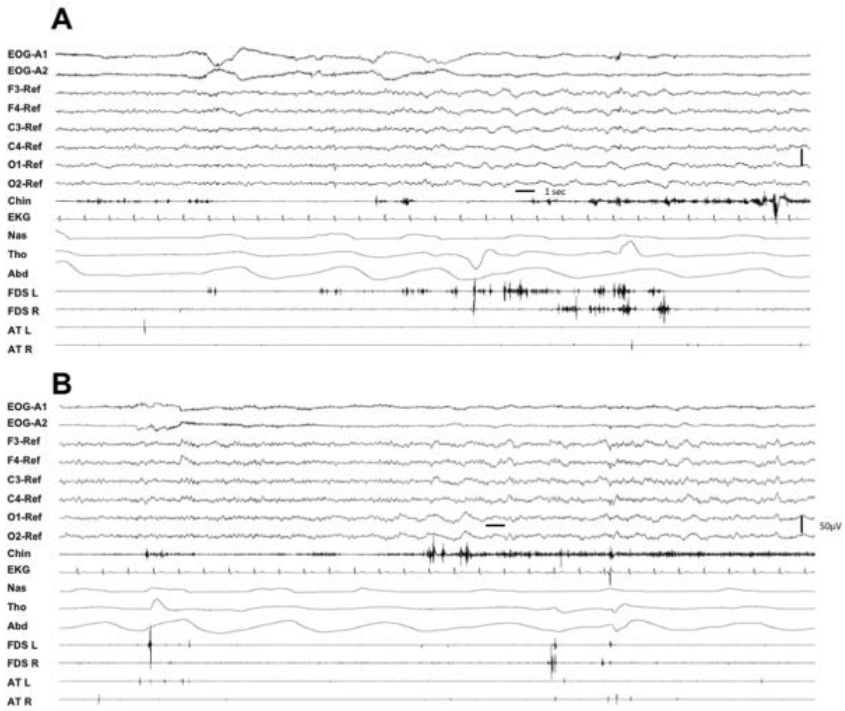


Abbreviations: EOG: electrooculogram; EEG: electroencephalogram referenced to both ears; Ment: electromyography of mentalis muscle; Note the calibration mark for time/EEG voltage.

In five out of the 26 (19.2%) patients in whom REM sleep was recorded during more than three minutes, the REM sleep periods showed delta slowing (1-1.5 Hz) with moderate amplitude (30-50 µV), either diffuse or predominantly in the occipital derivations (Figure 5). In three of these five cases, the delta activity decreased in amplitude when rapid eye movements appeared,

and in the remaining two the delta activity was unmodified during the entire duration of the REM sleep period. In all five patients the occipital background frequency during wakefulness was <8 Hz.

Figure 5. Intermittent delta slowing of moderate amplitude during REM sleep that is diffuse (A) or predominantly occipital (B).



Abbreviations: EOG: electrooculogram; EEG: electroencephalogram referenced to both ears; Ment: electromyography of the mentalis muscle; EKG: electrocardiogram; NAS: nasal air flow; THO: thoracic respiratory movement; ABD: abdominal respiratory movement; FDS: electromyogram of the left (L) and right (R) flexor digitorum superficialis; AT: electromyography of the left (L) and right (R) anterior tibialis. Note the calibration mark for time/EEG voltage. Note the excessive electromyographic activity in the mentalis muscle and limbs related to REM sleep behavior disorder.

Abnormal behavioral manifestations. During wakefulness, two patients had abnormal behaviors with the eyes closed, as soon as the lights were turned off for the start of the V-PSG study. In one of these two cases, V-PSG showed

the patient gesturing (picking non-existing objects from the air) with the eyes closed while the EEG showed diffuse slow theta/delta activity of moderate amplitude that was attenuated when the finalistic behaviors occurred (Video 1). This pattern of EEG activity was the same that the patient had during quiet wakefulness with the eyes closed and lights on. In this patient, V-PSG showed RBD. The other case had complex nocturnal visual hallucinations. She reported a six-year history of talking for hours during the night as soon as she laid down in bed and the lights were switched off. V-PSG showed complex motor and vocal manifestations that occurred with the eyes closed and included hand gesturing, kissing like movements, and speaking for three hours in what appeared to be a quiet but emotional conversation with someone about several topics such as a dance party, recipes and medical research (Video 2). During the speech she made intermittent pauses to apparently "listen to her partner". Simultaneous EEG during the speech episode showed alpha rhythm intermingled with theta activity (sometimes interrupted by glosso-kinetic artifacts due to talking). The background EEG activity of this patient during quiet wakefulness with the eyes closed and the lights on showed theta/delta slowing and no alpha rhythm. This patient did not achieve REM sleep during the V-PSG study. When asked the next day about this long speech episode she admitted experiencing well-defined visual and auditory hallucinations (an unknown person talking to her) with preserved insight and that she felt the need to interact with the hallucination by having a conversation about ordinary topics.

During the transition from wakefulness to sleep, one patient had an episode of hallucinatory-like behaviors, with the eyes closed, consisting in gesturing (picking imaginary objects from the air) and dialogue-like vocalizations while she intermittently snored (Video 3). The EEG during this episode showed continuous delta rhythm with superimposed bursts of FIRDA. This EEG pattern was different from the alpha rhythm that the patient had during quiet wakefulness with the eyes closed. The informant of this patient reported a

history suggestive of RBD but PSG excluded this parasomnia (rate of EMG activity in REM sleep was 16.9% without abnormal behaviors in REM sleep) and only showed abnormal behaviors during the wake-sleep transition.

Upon arousals from sleep, ten (31.2%) patients displayed abnormal behaviors that appeared to be acting out a dream (e.g., gesturing, talking, shouting) (Table 2). The most frequent manifestations were of short (<15 seconds) duration, and occurred at the end of obstructive apneic events (Video 4) and, less frequently, during prominent periodic limb movements. These short events arose from arousals occurring in the transition from wakefulness to stage N1, from stage N2 and from REM sleep.

Three patients (Table 2, patients 1, 2 and 10) displayed abnormal behaviors during arousals from REM sleep that were of short duration. They were not violent and consisted in vocalizations and gesturing (picking inexistent objects from the air). In two of these three patients, the trigger of the arousals was the end of an apneic event. None of these three patients had RBD.
In one patient, episodes of >15 seconds arose during spontaneous or noise-induced arousals from stage N3 (Table 2, patient 8). The episodes were clinically similar to the confusional arousals that are typically seen in NREM sleep parasomnias (e.g., opening the eyes and looking around, and talking as if the patient was threatened by someone) (Video 5). This patient had no past medical history of NREM sleep parasomnia or other sleep disorders.

The clinical history of one patient (Table 2, patient 10) was suggestive of RBD but V-PSG excluded this parasomnia (rate of EMG activity in REM sleep was 9.7% without abnormal behaviors in REM sleep) and demonstrated vocalizations during the arousals that occurred immediately after apneic events in REM sleep (the AHI in REM sleep was 55). In this patient, treatment with CPAP eliminated unpleasant dreams and dream-enacting behaviors.

Table 2. Abnormal manifestations during arousals arising from sleep

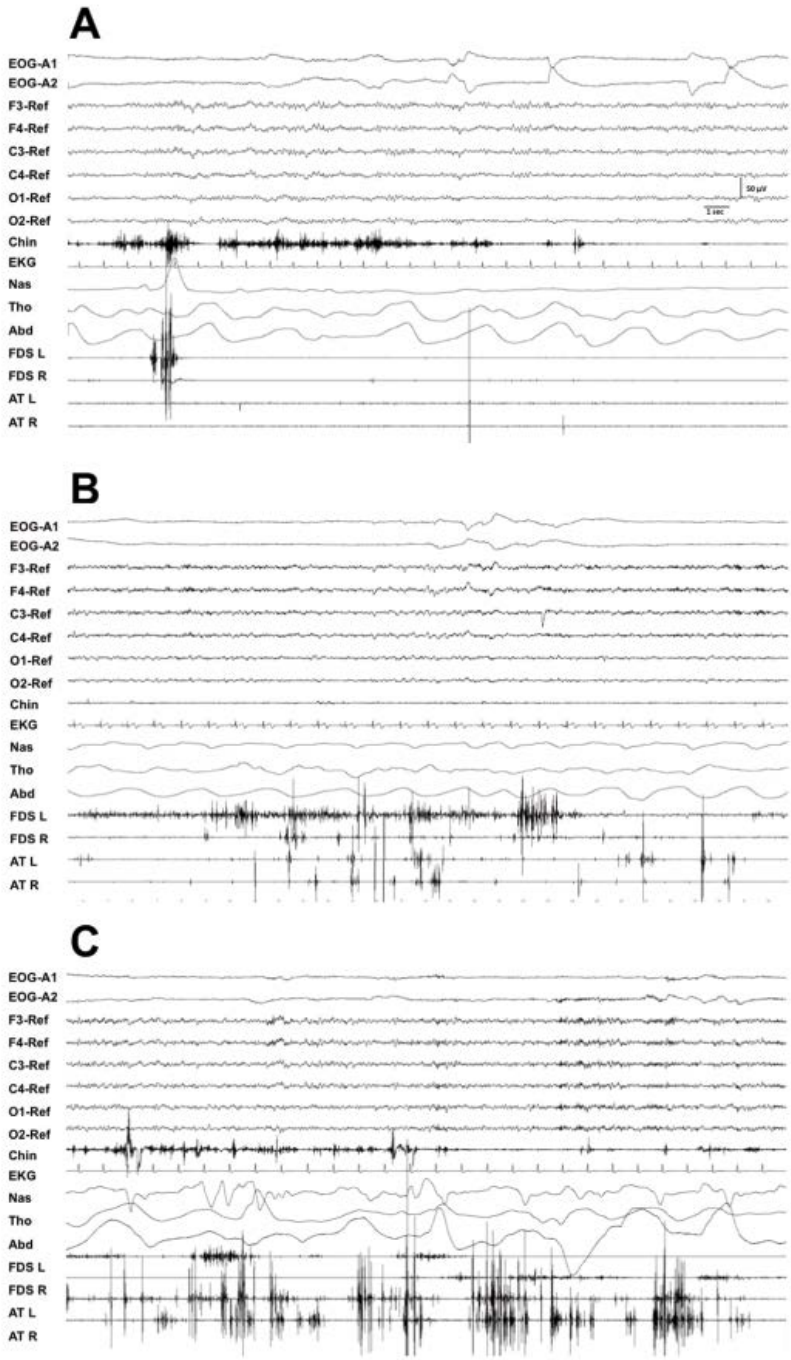
PATIENT, GENDER, AGE (Y)	PRECEDING STAGE	TRIGGER	DURATION OF THE BEHAVIOR (SEC)	STATE OF THE EYES	APPARENT EMOTION	TYPE OF MANIFESTATION (NUMBER OF EPISODES)
1,F,71	Transition from wakefulness to N1	None	3	Closed	Quiet	Gesturing (1), talking (1)
2,M,82	N2	End of apnea	2	Closed	Quiet	Raising the head (2), body jerking (5), moaning (2)
2,M,82	N2	End of apnea	6	Closed	Quiet	Whispering (2), moaning (1), talking (1), moving the head and legs(1), crossing fingers (1)
3,F,86	N2	End of apnea	3	Closed	Quiet	Vocalization (1), grabbing the sheet (1)
4,M,82	N2	End of apnea	4	Closed	Quiet	Moaning (3)
5,F,85	N2	Snore	3	Closed	Agitated	Shouting (1)
6,F,80	N2	End of apnea	5	Closed	Quiet	Vocalization (1)
7,M,74	N2	Periodic limb movements	5	Closed	Quiet	Gesturing (4), raising the arm (2), moving the arms and mouth (1)
8,M,76	N2	External noise	5	Open	Agitated	Talking (1), sitting up in bed (1)*
9,M,80	N2	None	12	Closed	Quiet	Touching the nose (1)
9,M,80	N2	None	14	Open	Quiet	Gesturing as “picking things”(1)*
8,M,76	N3	None	40	Open	Quiet	Looking around (1)
8,M,76	N3	External noise	45	Closed	Agitated	Talking such as threatening a person (1)*
8,M,76	N3	None	70	Open	Quiet	Touching the bed rails (1)
10,M,80	REM	End of apnea	1	Closed	Quiet	Vocalization (3)
2,M,82	REM	End of apnea	3	Closed	Quiet	Gesturing as “picking things” (1)
1,F,71	REM	None	14	Open	Quiet	Talking (1)*

M: Male; F: Female; *: Behaviors that resulted in an awakening.

The clinical history of another patient (Table 2, patient 7) was also suggestive of RBD which was excluded by V-PSG (rate of EMG activity in REM sleep was 8.5% without abnormal behaviors in REM sleep) that showed dream-enacting behaviors during the arousals that were linked to prominent periodic limb movements in NREM sleep involving not only the legs but also the whole body with a PLMS index of 68.

Diagnosis of RBD. RBD was found in 13 (50.0%) of the 26 patients in whom REM sleep was recorded during more than three minutes (Table 3). These were ten (76.9%) men and three (23.1%) women with a mean age of 77.4 ± 5.7 (range, 64-86) years, and a mean percentage of EMG activity in REM sleep of 53.7 ± 26.6 (range, 17.7-100) (Figure 6). Eleven (84.6%) out the 13 RBD patients had a clinical history suggestive of RBD, but eight (72.7%) had no recollection of their abnormal sleep behaviors and the relevant information had to be obtained from the caregiver. The two remaining RBD patients (Table 3, patients 6 and 7) were two men who were not aware of displaying dream-enacting behaviors and had no witness to describe their nocturnal sleep. Seven of the 13 (53.8%) patients with RBD recalled unpleasant dreams and five (38.5%) suffered injuries due to RBD episodes. RBD onset preceded cognitive decline in seven (63.6%) patients (six men, mean interval of 15.7 ± 6.2, range 8-25 years), coincided with cognitive impairment in three (27.3%) (one man and two women), and developed after the onset of cognitive decline in one (9.1%) man by five years. None of the patients and informants reported a temporal association between RBD onset and the introduction or withdrawal of a medication. In the 13 patients with RBD, the abnormal behaviors seen during REM sleep in the V-PSG were classified as severe in seven (53.8%), moderate in three (23.1%) and mild in three (21.3%) (Video 6).

Figure 6. REM sleep behavior disorder showing excessive electromyographic activity in REM sleep mainly in the mentalis muscle (A), only in the limb muscles (B) and in both mentalis and limb muscles (C).



Abbreviations: EOG: electrooculogram; EEG: electroencephalogram referenced to both ears; Ment: electromyography of the mentalis muscle; EKG: electrocardiogram; NAS: nasal air flow; THO: thoracic respiratory movement; ABD: abdominal respiratory movement; FDS: electromyogram of the left (L) and right (R) flexor digitorum superficialis; AT: electromyography of the left (L) and right (R) anterior tibialis. Note the calibration mark for time/EEG voltage.Abbreviations: RBD: REM sleep behavior disorder; V-PSG: Video-polysomnography; H-L: Hallucinatory-like behaviors; PLMD: Periodic limb movement disorder; OSAHS: Obstructive sleep apnea-hypoapnea syndrome; *: RBD was diagnosed in nine patients in the baseline study. In two patients RBD was diagnosed in a second study (one case because REM sleep was not attained in the baseline study and in the other case because REM sleep was normal in the baseline study); **: One patient did not achieve sleep during V-PSG and refused to undergo a second study. In one patient only 2.5 minutes of REM sleep were recorded and they refused to undergo a second study. In one patient REM sleep was not recorded in both the baseline and the second V-PSG study; ***: In two patients REM sleep was not recorded and both patients refused to undergo a second V-PSG study. In the remaining patient only 0.5 minutes of REM sleep were attained, and a second study was refused.

Table 3. Clinical and video-polysomnographic findings during REM sleep in the 13 patients with RBD

PATIENT	GENDER, AGE (Y)	CLINICAL HISTORY SUGGESTIVE OF RBD	SEVERITY OF BEHAVIORS DURING REM SLEEP*	BEHAVIORS IN REM SLEEP DURING V-PSG	EMG ACTIVITY IN REM SLEEP (%)**
1	M,79	Yes	Severe	Punching and kicking	71.4
2	M,76	Yes	Severe	Waving the arms vigorously, whole body jerks	65.9
3	M,80	Yes	Severe	Waving the arms vigorously, groaning	65.5
4	M,75	Yes	Severe	Kicking, screaming, gesturing, groaning	56.2***
5	M,86	Yes	Severe	Kicking	36.5
6	M,64	No	Severe	Gesturing and waving the arms vigorously, whole body jerks	20.8+
7	M,79	No	Severe	Kicking	17.7+
8	M,80	Yes	Moderate	Gesturing, moaning	100
9	M,78	Yes	Moderate	Gesturing, groaning, whole body jerks	71.5
10	M,72	Yes	Moderate	Talking, whole body jerks	70.7
11	F,72	Yes	Mild	Groaning, prominent limb jerks	46.2
12	F,83	Yes	Mild	Upper limb jerks	36.6
13	M,82	Yes	Mild	Limb jerks, whole body jerks	34.9

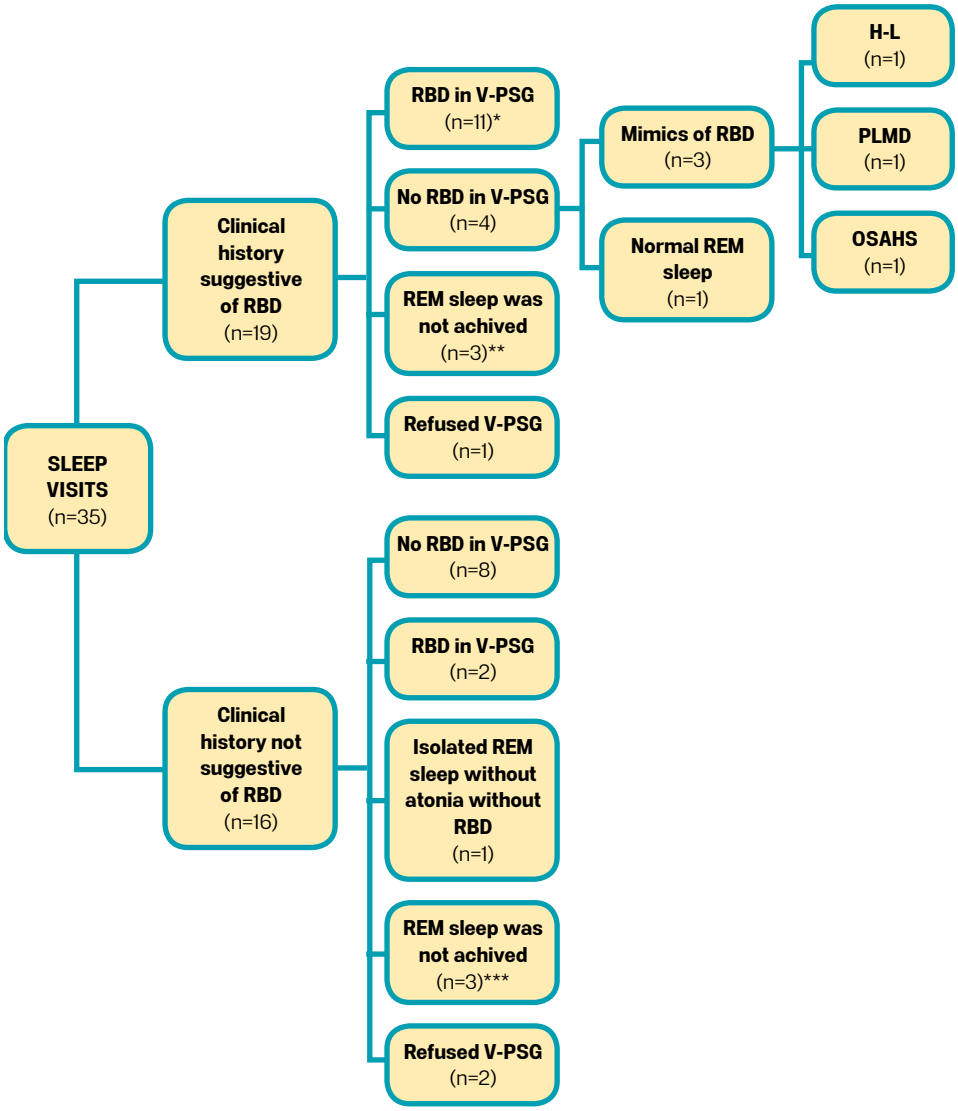
M:Male; F:Female; *: Severity of behaviors during REM sleep recorded during video-polysomography was assessed according to a system previously described in our center (reference 34); **: Percentage of combined phasic and tonic electromyographic activity in the mentalis muscle plus phasic electromyographic activity in the bilateral flexor digitorum superficialis measured in mini epochs of 3-seconds in the patients who achieved REM sleep without artifacts in the electromyographic leads (n=12); ***: Due to technical artifacts in the upper limb electromyographic leads, the activity could only be quantified in the mentalis muscle. Phasic and tonic electromyographic activity in the mentalis was above the cut-off value of 18.1% for the diagnosis of REM sleep behavior disorder (reference 32). Video analysis of this patient showed prominent abnormal behaviors in the extremities in REM sleep ; +: Quantification of EMG activity in REM sleep was below the cut-off of 32% but audiovisual recording showed abnormal manifestations typical of REM sleep behavior disorder.

RBD was diagnosed in ten patients in the baseline V-PSG study. RBD could not be diagnosed in three cases in the baseline study because REM sleep was not recorded y in one, REM sleep lasted less than 10 minutes in another, and for the third case, the baseline study showed normal REM sleep while the clinical history was highly suggestive of RBD and other causes of dream-enactment were not detected in the V-PSG. For all three, RBD was diagnosed in a second V-PSG study.

Three of the five patients who were treated with clonazepam prior to the study because their physicians suspected RBD, were confirmed to have RBD by V-PSG. Of the remaining two patients, one had normal REM sleep and no abnormal manifestations disclosed in the V-PSG study, and the other was diagnosed with obstructive sleep apnea mimicking RBD symptomatology (Table 2, patient 10).

According to the clinical history obtained by the sleep expert, 19 (54.3%) out of the 35 DLB patients evaluated had a history suggestive of RBD (Figure 7). In these 19 subjects, REM sleep was recorded in 15 (78.9%) and RBD was confirmed in 11 (73.3%). The remaining four subjects did not have RBD but had abnormal conditions seen outside REM sleep that mimicked RBD (one with hallucinatory-like behaviors in the transition from wakefulness to sleep, one with arousals related to prominent periodic body movements, and one with apneas related arousals; Table 2, patients 1, 7 and 10 respectively) and one had normal REM sleep without abnormal movements and without excessive EMG activity. Two patients with RBD confirmed by V-PSG (Table 3, patients 6 and 7) had no clinical history suggestive of RBD (patients did not recall displaying abnormal sleep behaviors and had no bed partners to provide additional information) (Figure 7).

Figure 7. Video-polysomnographic findings and diagnosis of REM sleep behavior disorder in patients with and without clinical history suggestive of this parasomnia.



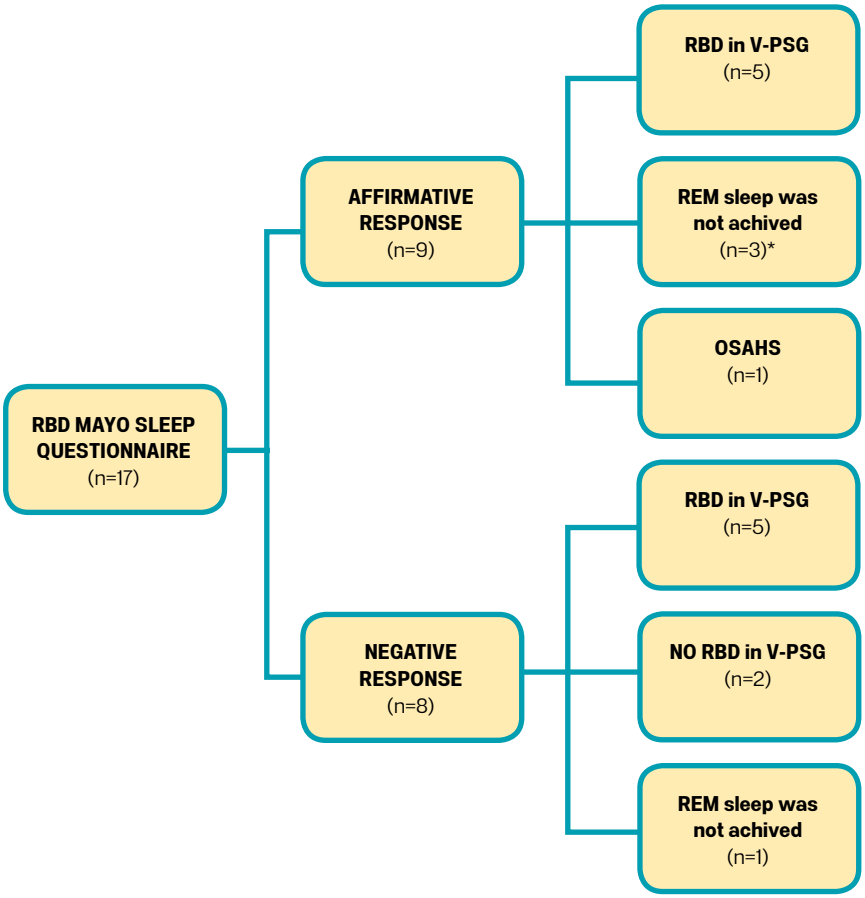
We identified one patient with *isolated REM sleep without atonia without RBD* in whom the clinical history was not suggestive of RBD but V-PSG showed excessive EMG activity in REM sleep (37.7%) without associated abnormal behaviors (Figure 7).

RBD could not be confirmed or excluded in three patients with a suggestive clinical history of this parasomnia. Two of these patients did not attain REM sleep and in one, REM sleep was less than three minutes. All were taking antidepressants. One accepted to undergo a second study but REM sleep was still not recorded and the other two refused the second study.

For the diagnosis of RBD, sensitivity of the clinical history taken by the sleep expert was 84.6%, specificity 66.7%, positive predictive value 73.3%, and negative predictive value 81.8%.

The MSQ could only be administrated to 17 informants; nine (52.9%) responded affirmatively and eight (47.1%) negatively (Figure 8). Among the nine informants who gave an affirmative answer, five patients had RBD, in another patient the V-PSG showed normal REM sleep but obstructive sleep apnea mimicking RBD symptoms, and in the remaining three patients REM sleep was not attained. Of the eight patients whose informants gave a negative answer, five had RBD, RBD was excluded in two, and REM sleep was not achieved in one. For the diagnosis of RBD, sensitivity of the MSQ was 50%, specificity 66.7%, positive predictive value 83.3%, and negative predictive value 28%.
Between patients with and without RBD, there were no differences in demographic and clinical data and in medication use. Compared with patients without RBD, those with RBD had slower sleep spindle frequency and a higher rate of EMG activity in REM sleep (as expected). The RBD group had more patients without sleep spindles and delta EEG activity in REM sleep compared to the group without RBD (Table 4).

Figure 8. Video-polysomnographic findings and diagnosis of REM sleep behavior disorder according to the answer of the Mayo sleep questionnaire



Abbreviations: RBD: REM sleep behavior disorder; V-PSG: Video-polysomnography; OSAHS: Obstructive sleep apnea-hypoapnea syndrome; *: One patient did not attain any sleep during V-PSG and refused to undergo a second study. In one patient only 2.5 minutes of REM sleep were recorded and refused to undergo a second study. In one patient REM sleep was not recorded in both the baseline and the second V-PSG study.

Table 4. Demographic, clinical and sleep characteristics of patients with and without REM sleepbehavior disorder

	RBD	NO RBD	P VALUE
Patients, (n)	13	13	
Male sex, n (%)	10 (76.9)	6 (46.2)	0.226
Age at V-PSG, y	77.4 ± 5.7 (64.0-86.0)	78.4 ± 6.2 (69.0-86.0)	0.672
Age at DLB diagnosis, Y	76.2 ± 4.9 (64.0-83.0)	76.8 ± 5.8 (67.0-85.0)	0.774
Visual hallucinations, n (%)	8 (61.5)	9 (69.2)	1.000
Fluctuations, n (%)	10 (76.9)	6 (46.2)	0.226
Parkinsonism, n (%)	12 (92.3)	9 (69.2)	0.322
Epworth Sleepiness Scale score, n	8.9 ± 3.7 (4-16)	10.2 ± 6.7 (1-24)	0.595
Benzodiazepines, n (%)	4 (30.8)	6 (46.2)	0.688
Antidepressants, n (%)	3 (23.1)	6 (46.2)	0.411
Cholinesterase inhibitors, n (%)	8 (61.5)	10 (76.9)	0.673
Dopaminergics, n (%)	4 (30.8)	4 (30.8)	1.000
Neuroleptics, n (%)	3 (23.1)	4 (30.8)	1.000
Occipital EEG frequency in wakefulness, n	7.8 ± 1.7 (5.0-12.0)	8.7 ± 9.7 (6.5-10.0)	0.132
Decreased reactivity in wakefulness*, n (%)	8 (61.5)	4 (30.8)	0.238
Sleep efficiency, (%)	69.8 ± 16.3 (41.0-94.5)	64.8 ± 20.3 (22.0-94.0)	0.420
Total sleep time, (min)	323.2 ± 68.5 (190.0-414.0)	306.8 ± 94.8 (98.0-432.0)	0.619
Sleep onset latency, (min)	20.7 ± 18.6 (0.5-61.0)	42.3 ± 49.2 (2.5-163-0)	0.151
Sleep spindle frequency, (n)	11.2 ± 1.3 (9.5-14.0)	12.8 ± 1.4 (10.0-14.0)	0.013

	RBD	NO RBD	P VALUE
Absence of sleep spindles, n (%)	4 (30.8)	0 (0)	0.035
Arousal index, (n)	18.5 ± 12.2 (2.2-41.7)	30.2 ± 20.3 (3.2-71.2)	0.088
Awakenings, (n)	8.3 ± 16.2 (0-27.0)	6.9 ± 2.4 (0-59.5)	0.788
AHI, (n)	20.2 ± 18.8 (0-37.4)	14.9 ± 12.6 (1.0-59.6)	0.430
CT90, (%)	11.1 ± 23.8 (0-80.0)	13.7 ± 25.1 (0-90.0)	0.803
PLMSI, (n)	38.1 ± 47.4 (0-136.0)	23.8 ± 25.0 (0-67.6)	0.348
Delta activity in REM sleep, n (%)	5 (38.5)	0 (0.0)	0.020
REM sleep latency, (min)	118.8 ± 85.4 (51.5-321.5)	214.4 ± 104.8 (74.5-430.0)	0.018
REM sleep, (min)	45.3 ± 31.5 (14-106.5)	43.8 ± 26.2 (9.5-94.0)	0.891
REM sleep, (%)	15.5 ± 9.3 (3.9-28.5)	13.9 ± 7.0 (4.5-27.2)	0.623
Phasic and tonic EMG activity in the mentalis plus phasic EMG activity in the FDS in REM sleep**, (%)	53.1 ± 24.7 (17.7-100)	13.2 ± 10.1 (2.8-37.2)	<0.001

Values are expressed as mean ± standard deviation, number, frequency and range; *: Eye opening partial elicited or no changes in the occipital dominant activity; **: Activity measured in 3-second mini epochs; AHI: Apnea-hypoapnea index; CT90: Percentage of sleep time with oxyhemoglobin saturation below 90%; PLMSI: Periodic leg movements in sleep index; RBD: REM sleep behavior disorder; EMG: Electromyography; FDS: Flexor digitorum superficialis.

There were no demographic, clinical or medication use differences between patients with slow (<8Hz) and alpha (8-12 Hz) occipital rhythm during wakefulness. Those with slow frequency when awake more often had decreased reactivity in wakefulness, slower sleep spindle frequency, absent sleep spindles, increased delta EEG activity in REM sleep, higher prevalence of RBD diagnosis, and greater rate of EMG activity in REM sleep (Table 5). There was a negative correlation between the occipital EEG frequency during wakefulness and the EMG activity in the mentalis during REM sleep ($r=-0.542$; $p=0.004$) and a tendency towards significance when the mentalis EMG activity was combined with the FDS in REM sleep ($r=-0.363$; $p=0.07$) (Figure 9).

Figure 9. Correlation between the electromyographic activity in REM sleep and the frequency of the occipital electroencephalographic activity during wakefulness.

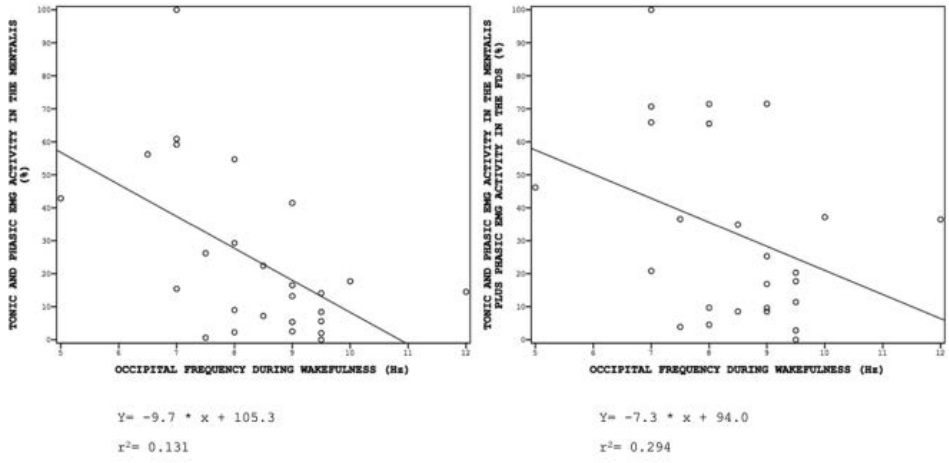


Table 5. Demographic, clinical and sleep characteristics according to the occipital frequency of the electroencephalographic occipital activity during wakefulness

	<8 HZ	≥8 HZ	P VALUE
Patients, (n)	11	21	
Male sex, n (%)	7 (63.6)	13 (61.9)	1.000
Age at V-PSG, y	75.8 ± 6.0 (64.0-85.0)	79.5 ± 4.9 (69.0-86.0)	0.075
Age at DLB diagnosis, Y	75.3 ± 5.8 (64.0-84.0)	77.7 ± 4.4 (67.0-85.0)	0.204
Visual hallucinations, n (%)	7 (63.6)	15 (71.7)	0.703
Fluctuations, n (%)	7 (63.6)	14 (66.7)	1.000
Parkinsonism, n (%)	9 (81.8)	18 (85.7)	1.000
Epworth Sleepiness Scale score, n	11.3 ± 5.6 (5-24)	8.8 ± 5.1 (1-18)	0.229
Benzodiazepines, n (%)	2 (18.2)	10 (47.6)	0.139
Antidepressants, n (%)	3 (27.3)	10 (47.6)	0.450
Cholinesterase inhibitors, n (%)	9 (81.8)	15 (71.4)	0.681
Dopaminergics, n (%)	5 (45.5)	5 (23.8)	0.252
Neuroleptics, n (%)	3 (27.3)	4 (19.0)	0.667

	<8 HZ	≥8 HZ	P VALUE
Occipital EEG frequency in wakefulness, n	6.7 ± 0.7 (5.0-7.5)	9.2 ± 1.1 (8.0-12.0)	<0.001
Decreased reactivity in wakefulness*, n (%)	10 (90.9)	4 (19.0)	<0.001
Sleep efficiency, (%)	66.4 ± 17.6 (41.0-94.5)	64.0 ± 22.0 (6.0-94.0)	0.706
Total sleep time, (min)	305.2 ± 74.8 (190.0-414.0)	300.5 ± 103.9 (26.5-432.0)	0.898
Sleep onset latency, (min)	18.1 ± 20.4 (0.5-61.0)	48.0 ± 58.2 (2.5-233.5)	0.129
Sleep spindle frequency, (Hz)	11.1 ± 1.0 (10.0-12.0)	12.5 ± 1.4 (9.5-14.0)	0.029
Absence of sleep spindles, n (%)	4 (40.0)	0 (0.0)	0.002
Arousal index, (n)	21.2 ± 13.2 (3.2-42.5)	28.5 ± 18.6 (2.2-71.2)	0.274
Awakenings, (n)	6.5 ± 6.8 (0-17.0)	8.3 ± 14.1 (0-59.5)	0.707
AHI, (n)	12.5 ± 8.5 (0.2-23.9)	22.0 ± 18.3 (0-59.6)	0.152
CT90, (%)	12.7 ± 31.4 (0-90.0)	10.5 ± 17.9 (0-80.0)	0.816
PLMSI, (n)	42.2 ± 53.0 (0.7-136)	23.8 ± 25.0 (0-67.6)	0.099
Delta activity in REM sleep, n (%)	5 (55.5)	0 (0.0)	0.001
REM sleep latency, (min)	179.5 ± 140.4 (51.5-430.0)	184.8 ± 106.2 (69.0-397.5)	0.911
REM sleep, (min)	45.5 ± 38.0 (0.5-106.5)	41.9 ± 23.4 (2.5-94.0)	0.757
REM sleep, (%)	14.3 ± 10.8 (0.5-28.5)	14.2 ± 7.1 (0.7-27.2)	0.986
RBD, n (%)	7 (77.8)	6 (33.3)	0.046
Phasic and tonic EMG activity in the mentalis in REM sleep**, (%)	45.2 ± 31.2 (0.6-100)	14.8 ± 14.5 (14.8-14.5)	0.002
Phasic and tonic EMG activity in the mentalis plus phasic EMG activity in the FDS in REM sleep**, (%)	49.2 ± 32.5 (3.9-100)	25.1 ± 23.3 (0-25.1)	0.049

Values are expressed as mean ± standard deviation, number, frequency and range; *: Eye opening partial elicited or no changes in the occipital dominant activity; **: Activity measured in 3-second mini epochs; AHI: Apnea-hypoapnea index; CT90: Percentage of sleep time with oxyhemoglobin saturation below 90%; PLMSI: Periodic leg movements in sleep index; RBD: REM sleep behavior disorder; EMG: Electromyography; FDS: Flexor digitorum superficialis.

Discussion

In this prospective study, we describe a wide range of sleep-wake abnormalities in a consecutive cohort of patients with a diagnosis of DLB with mild dementia severity. Patients were enrolled irrespective of the presence of sleep complaints. Clinical assessment, sleep questionnaires and detailed V-PSG analysis allowed us to provide a complete portrait of sleep in DLB. This included a comprehensive description of the EEG abnormalities seen in wakefulness and sleep, the types and characteristics of abnormal behaviors occurring at night, and the clinical and V-PSG characteristics of RBD. In addition, our study examined the utility of the clinical history, the MSQ and the V-PSG for the identification of RBD, a parasomnia that currently represents a core clinical feature for the diagnosis of DLB (13).

Sleep-related symptomatology

Our work confirms previous studies showing that sleep complaints are frequent in DLB and include a wide range of abnormalities, namely overall subjective poor sleep quality, sleep onset and maintenance insomnia, early awakenings, loud snoring, witnessed apneas, hypersomnia, restless leg syndrome, nocturnal visual hallucinations, unpleasant dreams and abnormal behaviors at night (121–123,128–130,134). Our study also confirms that V-PSG in DLB commonly reveals reduced mean sleep efficiency, a high number of patients with elevated number of obstructive apneas, PLMS and RBD (128–130,133,134). Paradoxically, only a minority of the patients sought medical help for their sleep problems.

The origin of the sleep disturbances in DLB is complex and heterogeneous and may be related to several factors including aging, the effect of some medications on sleep (e.g., antidepressants, benzodiazepines, neuroleptics), coexisting abnormalities (e.g., parkinsonism, hallucinations), depres-

sion, and the neurodegenerative process damaging the areas that generate and maintain sleep.

EEG activity during wakefulness and sleep

Abnormal sleep-wake architecture was frequent in DLB and occurred in 75% of the patients. Abnormalities were **1)** slow occipital activity in the theta range during wakefulness occasionally combined with delta waves, **2)** persistence during sleep onset of occipital alpha/theta activity at least 1 Hz slower than the dominant occipital rhythm seen during wakefulness, **3)** FIRDA at sleep onset, **4)** semi-rapid eye movements and sustained EMG activity at sleep onset, **5)** transient sharp waves in NREM sleep, **6)** sleep spindles of low frequency in stage N2, **7)** absence of sleep spindles and K complexes in NREM sleep, **8)** diffuse or occipital predominant delta slowing of moderate amplitude during REM sleep and, 9) REM sleep without muscle atonia. These findings expand previous studies in DLB that showed posterior slow-wave activity, FIRDA, transient slow or sharp waves, and decreased reactivity with eye opening during wakefulness (211,219,220). Similar to the current study, two publications described abnormalities in the sleep architecture in DLB consisting in, as defined by the authors, dissociated sleep (simultaneous presence of elements of wakefulness, NREM and REM sleep) and *ambiguous sleep* (diffuse slow-wave activity alternating with REM sleep without atonia) (129,221).

Most of the sleep-wake architecture alterations described in the present study in DLB were reported in our series of patients with advanced Parkinson disease and dementia (222). For example, in Parkinson disease the sleep spindle density is reduced and this is indicative of the development of dementia (223–225). The absence of sleep spindles and sleep spindles of low frequency may represent a marker of cholinergic thalamo-cortical dysfunction in both DLB and Parkinson disease. During normal stage N2, the brainstem

sends cholinergic projections to the reticular nucleus of the thalamus which, in turn, generates sleep spindles (226). Patients with DLB, Parkinson disease and isolated RBD show cholinergic denervation on the thalamus (227).

The sleep-wake abnormalities we detected challenged the identification of wakefulness, sleep, and the sleep stages. Sleep onset was difficult to identify in patients lacking alpha rhythm and showing dominant slow EEG activity during wakefulness. Identification of NREM sleep was also difficult in subjects lacking vertex waves, K complexes and sleep spindles (or with sleep spindles of low frequency). Instead of forcing these sleep epochs to be scored into one of the conventional NREM sleep stages (N1, N2 and N3) we used the term undifferentiated NREM sleep to describe NREM sleep that was without the typical elements of this well-differentiated sleep stage. REM sleep was not easily identified in subjects with delta EEG activity and absence of muscular atonia.

Taken together, our observations highlight that scoring sleep in patients with neurodegenerative diseases (particularly with dementia) can be challenging because the main parameters used to score sleep (EEG, EOG and mentalis EMG) are often altered. In these patients, rather than forcing their sleep architecture to be scored into the standard system that was originally delineated for non-neurologically impaired subjects, a new more descriptive system is required with new rules using a descriptive approach (212).

Abnormal behavioral manifestations

The use of V-PSG permitted the detection of abnormal behaviors occurring not only in REM sleep (resulting from RBD) but also during wakefulness, the transition from wakefulness to sleep, NREM sleep and upon arousals. Behaviors consisted in 1) hallucinatory-like manifestations occurring during wakefulness and in the transition from wakefulness to sleep (always

with the eyes closed and the lights turned off), 2) arousal behavioral-related episodes emerging from both NREM and REM sleep, and 3) RBD. The clinical manifestations among these categories were very similar and included talking, moaning, yelling, raising the head, gesturing, sitting up in bed, and jerking. The abnormal behaviors arising from arousals were spontaneous or triggered by apneic events, periodic limb movements and external noises. Our findings confirm and extend previous reports underscoring the difficulties in identifying the nature of the abnormal behaviors displayed in DLB using only the clinical history (129,130,221). V-PSG was essential to detect and characterize these behavioral manifestations. This is important because hallucinations, confusional arousals, obstructive sleep apnea, periodic limb movements and RBD are treatable conditions that each requires very different therapeutic approaches (e.g., antipsychotics, CPAP, dopaminergics, clonazepam). In fact, in two patients treated with clonazepam because their symptoms resembled RBD, V-PSG ruled out this parasomnia and showed dream-enacting behaviors elicited by obstructive apneic events in one case and normal REM sleep in the other.

Diagnosis of RBD

RBD was diagnosed in 50% of the patients using a detailed V-PSG analysis in which the EMG activity in REM sleep was manually quantified and audiovisual recordings were carefully analyzed. According to our data, the prevalence of RBD in DLB is much higher than the prevalence of RBD reported in Alzheimer disease (<5%) (228), similar to Parkinson disease (40%-50%) (229–231) and lower than in multiple system atrophy (90-100%)49. Of note, our cohort comprised consecutive patients with a recent diagnosis of DLB and mild dementia severity who were recruited irrespective of the presence of sleep complaints. The few studies that have examined the prevalence of RBD in DLB using PSG have reported rates ranging from 38% to 100% (126,128,129,134).This wide range of frequencies may

be attributable to methodological aspects, referral bias (consecutive patients vs. patients referred because of dream-enacting behaviors), sample bias (patients with mild dementia severity vs. patients with all degrees of dementia severity; patients with recent diagnosis of DLB vs. patients with advanced disease), and criteria used to diagnose RBD (manual quantification of EMG using normative cut-off values vs. clinical impression of increased EMG activity; audiovisual analysis vs. lack of audiovisual analysis).

Some studies have shown that DLB is more prevalent in men while others have reported a female predominance (200,203). In the present study, we found a male predominance of RBD in DLB (77%) that was higher than the percentage of males who were enrolled in the study (66%). Most of the RBD patients and their caregivers reported that the symptoms of RBD preceded, by several years, the onset of cognitive decline. This is in concordance with the findings from our cohort of patients with isolated RBD; of 261 isolated RBD subjects (78.5% males), 44 (16.9%) have converted to DLB and of these patients, 93.2% were male with a mean interval from estimated RBD onset to DLB diagnosis of 12.0 ± 4.6 (range, 4.8-22.4) years, and a mean interval from RBD diagnosis by V-PSG to DLB diagnosis of 6.3 ± 3.4 (range, 0.13-14.83) years (unpublished data). Other features of RBD in DLB that deserve to be mentioned are the lack of unpleasant dream recall in 46%, and not being aware of displaying RBD-related sleep behaviors in 69% of the patients. In these instances, witness statements were crucial by raising suspicion for RBD.

Compared with patients without RBD, those with RBD had a higher rate of EMG activity in REM sleep (as expected), more often had delta EEG activity in REM sleep and had more abnormalities in NREM sleep, suggesting a more widespread neurodegenerative process. A new finding from our study was that DLB patients with slow (<8Hz) occipital EEG activity during wakefulness more frequently had RBD than those who generated alpha rhythm.

The association between RBD and slow EEG activity during wakefulness was previously described in subjects with isolated RBD who later developed mild cognitive impairment and in patients with Parkinson disease (232,233). Our study is the first, however, to report a correlation between the awake occipital EEG frequency and the rate of EMG activity in REM sleep. This association in patients with DLB of mild severity may be explained by impairment of the subcortical cholinergic areas that innervate the cortex and are close to the brainstem areas generating REM sleep. The main sources of cholinergic input to the cortex are from the nucleus basalis of Meynert in the basal forebrain and the pedunculopontine/laterodorsal tegmental nucleus in the brainstem (234) . Impairment of these projections could produce the awake occipital EEG background slowing as well as the impairment in NREM and REM sleep architecture. Postmortem studies have shown that the nucleus basalis of Meynert and the pedunculopontine/laterodorsal tegmental nucleus area are damaged in patients with 1) isolated RBD (202,235), 2) isolated RBD that developed mild cognitive impairment (236), 3) isolated RBD that developed DLB and Parkinson disease (237), and 4) DLB (238). In DLB there is a prominent reduction in acetylcholinesterase activity within the occipital cortex (239) and it is known that anticholinesterase inhibitors can increase the frequency of the EEG during wakefulness (240).

In 2005, diagnostic criteria for DLB included visual hallucinations, parkinsonism, and fluctuations as the three core clinical features. RBD, confirmed by PSG, was considered one of the suggestive features because "(RBD) has been demonstrated to be more significantly frequent (in DLB) than in other dementing disorders" (11). In 2017, the diagnostic criteria were modified and a clinical history suggestive of RBD was included as the fourth core clinical feature (13). Confirmation of RBD with V-PSG was not required "due to the expense and lack of availability of PSG in many clinical settings" (241). Our current study shows, however, that in some cases the MSQ and the clin-

ical history were not able to distinguish RBD from other conditions. For the diagnosis of RBD, the MSQ showed low sensitivity (50%) and low negative predictive value (28%). Indeed, the original article that validated the MSQ showed a specificity of 72% because a number of patients that gave an affirmative response for RBD, were found to have other sleep disorders. Another limitation of the MSQ is that it requires input from a reliable informant, a situation that was not possible in 51% of the cases in our study. This is relevant since, as shown in our study, several DLB patients with true RBD were not aware of displaying abnormal behaviors at night and did not recall nightmares. The conditions that mimicked RBD were severe obstructive sleep apnea, prominent periodic limb movements in sleep and hallucinatory-like behaviors. Distinguishing these mimics from RBD is important because they have different pathophysiologic mechanisms and treatments, and according to the current criteria for DLB, may lead to an erroneous diagnosis of this disease (13). For example, a patient with Alzheimer disease can fulfill current diagnostic criteria of DLB if in addition to dementia and hallucinations (features that can be found in both DLB and Alzheimer disease) there is a clinical history suggestive of RBD that actually corresponds to other type of sleep disorder. V-PSG is the only instrument that can differentiate RBD from its mimics by showing excessive EMG activity in REM sleep linked to abnormal sleep behaviors. However, as shown in our study, V-PSG has some limitations that include 1) refusal to undergo the test, and 2) not achieving enough REM sleep for RBD to be properly evaluated. The latter may be a result of DLB patients having low sleep efficiency (first night effect) and that they usually take antidepressants (a medication that decreases the REM sleep time). When we asked our patients to undergo a second V-PSG study some declined and others accepted but again, did not attain REM sleep.

Our study has some limitations such as the relatively small number of patients enrolled, the refusal of more than 50% of the patients who were in-

vited to participate, the lack of a reliable informant about the patient's sleep in 51% of the cases, the use of medications such as antidepressants that can modify the sleep architecture, the lack of a healthy control group, and the lack of pathological confirmation of the diagnosis of DLB. These limitations were difficult to overcome in a study aimed at evaluating consecutive patients irrespective of the presence or absence of sleep complaints. The main strengths of our study are the comprehensive analyses of the patients' sleep complaints, the use of validated sleep scales and questionnaires, and performing V-PSG to describe the abnormal behaviors occurring at night as well as to diagnose RBD and detect mimics. Our work included the quantification of the EMG activity in REM sleep using normative values and meticulous audiovisual analysis of the behaviors detected.

In summary, our findings show that sleep-wake disturbances are common and complex in patients with DLB and that V-PSG is essential to diagnose RBD and rule out mimics. We report for the first time an association between the occipital EEG activity awake and the rate of EMG activity in REM sleep suggesting that both abnormalities may have a common subcortical origin in DLB.

Disclosure Statement

All the authors of the present study declare no conflict of interest concerning the manuscript.
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5. CHAPTER V: DIFFERENT PATTERN OF CSF GLIAL MARKERS BETWEEN DEMENTIA WITH LEWY BODIES AND ALZHEIMER'S DISEASE

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Tittle: Different pattern of CSF glial markers between dementia with Lewy bodies and Alzheimer's disease

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Key words: glial biomarkers, DLB, Lewy bodies, neuroinflammation, neurodegeneration

Abstract

The role of innate immunity in dementia with Lewy bodies (DLB) has been little studied. We investigated the levels in cerebrospinal fluid (CSF) of glial proteins YKL-40, soluble TREM2 (sTREM2) and progranulin in DLB and their relationship with Alzheimer's disease (AD) biomarkers. We included patients with DLB (n=37), prodromal DLB (prodDLB, n=23), AD dementia (n=50), prodromal AD (prodAD, n=53), and cognitively normal subjects (CN, n=44). We measured levels of YKL-40, sTREM2, progranulin, Aβ1-42, total tau (t-tau) and phosphorylated tau (p-tau) in CSF. We stratified the group DLB-prodDLB according to the ratio t-tau/Aβ1-42 (≥0.52, indicative of AD pathology). YKL-40, sTREM2 and progranulin levels did not differ between DLB groups and CN. However, those patients with DLB or prodDLB with a CSF profile suggestive of AD copathology had higher levels of YKL-40, but not sTREM2 or PGRN, than those without. Of these glial markers, only YKL-40 correlated with t-tau and p-tau in DLB and in prodDLB. YKL-40, sTREM2 and PGRN are not increased in the CSF of DLB patients. YKL-40 is only increased in DLB patients with an AD biomarker profile, suggesting that the increase is driven by the AD pathology. These data suggest a differential glial activation between DLB and AD.

Background

Epidemiological, pathological and genetic studies support the importance of the innate immunity in the pathophysiology of neurodegenerative diseases such as Alzheimer's disease (AD) and Parkinson's disease (PD) (136,138). In particular, astroglia and microglia play an important role in neurodegeneration (139,242). These two cellular types have very different functions in the central nervous system (CNS): microglia, the resident monocytic cells in the CNS, phagocyte cellular debris and protein aggregates, while astrocytes support neuronal and synaptic activities among other key functions (139,242) .

YKL-40 protein, also known as chitinase 3-like 1 protein, is a glycoprotein expressed by astrocytes near amyloid plaques in AD human brain (165,166). YKL-40 can be detected in cerebrospinal fluid (CSF) and the levels are increased in preclinical and prodromal AD, as well as in other neurodegenerative conditions, such as Frontotemporal Lobar Degeneration (FTLD), Amyotrophic Lateral Sclerosis (ALS) or Multiple Sclerosis (MS) (167,243–245).

In addition, other studies have implicated the triggering receptor expressed on myeloid cells 2 (TREM2) receptor in neurodegenerative diseases (246). Rare heterozygous variants in TREM2 have been linked with an increased risk of AD (142,247). Furthermore, recent studies have shown an elevation in the CSF of the soluble fragment of TREM2 (sTREM2) in early stages of sporadic AD (169,171,248) as well as in autosomal dominant AD (172).

Another line of evidence that support the role of inflammation in neurodegenerative conditions implicates the Progranulin protein (PGRN). PGRN is expressed in many tissues and cell types, where it is involved in angiogenesis, wound repair, cell proliferation, and inflammation (145). PGRN levels are decreased in CSF and blood of patients with heterozygous mutations in

the granulin gene (GRN), linked to FTLD with TAR-DNA-binding protein 43 inclusions (249–252). Furthermore, genetic variants that modulate GRN expression, such as the GRNrs5848 polymorphism, have been associated with an increased risk of AD (146,148).

There are multiple evidences that support that glia is activated in synucleinopathies (158). In particular, activated microglia targeting dopamine nigral neurons has been described in PD (159). Microglial activation in PD and dementia with Lewy bodies (DLB) has been implicated in the initiation and progression of the disease by means of secretion of pro-inflammatory cytokines and reactive oxygen species (158). In addition, synuclein released from neurons in PD and DLB can be endocytosed by astrocytes forming glial inclusions (159–161). These inclusions can induce changes in gene expression in astroglia, enhancing the inflammatory response and promoting neurodegeneration (159,161).

In this study, we investigated the CSF profile of YKL-40, sTREM2, PGRN in patients with DLB and prodromal DLB, and compared this pattern with that of AD. We also examined the influence of concomitant AD pathology in DLB on these biomarkers.

Results

Demographics and core CSF biomarkers

Table 1 summarizes the demographics and CSF biomarkers values of all the study participants. There were no significant differences between groups in gender, but CN subjects were significantly younger than the other groups. As expected, MMSE scores were lower in DLB and AD than in CN or groups with prodAD and prodDLB. Core AD CSF biomarkers also differed between groups (Table 1). DLB groups had lower levels of Aβ1-42 than CN but higher than AD groups. DLB groups had also higher levels of tau and p-tau than CN, but lower than AD groups. Frequency of APOEε4 allele in DLB groups was similar to CN and lower than AD groups ($p= 0.001$).

Relationship between glial biomarkers, age, APOE and clinical measures

There was no association between gender and any of the three glial markers, but there was a trend towards higher levels of sTREM2 in males ($p =0.06$). Therefore, all sTREM2 analyses were adjusted by gender. Age significantly correlated with CSF levels of YKL-40 and sTREM2 ($r = +0.351$; $p < 0.001$ and $+0.212$; $p < 0.006$, respectively) in the whole sample as previously reported (169,172). We did not find differences in the levels of any of the glial markers between APOEε4 carriers and non-carriers.

Table 1. Demographic and CSF biomarker data.

		CN (n = 44)	DLB (n = 37)	PRODROMAL DLB (n = 23)
	Age, y ± SD[a]	67.4 ± 5.1	76.5 ± 5	76.5 ± 6.4
	Sex, Female % (n)	56.8 (25)	54.1 (20)	56.5 (13)
	APOEε4, %* (n)[b]	18.2 (8)	24.3 (9)	34.8 (8)
	MMSE ± SD [c]	28.9 ± 1.2	23 ± 4.6	26.1 ± 2.4
Core AD biomarkers	**CSF Aß1-42, pg/mL ± SD [d]**	918.2 ± 212.2	602.7 ± 269.2	634 ± 197.7
	CSF t-tau, pg/mL ± SD [e]	228.8 ± 52.3	448.9 ± 333.9	371.3 ± 174.5
	CSF p-tau, pg/mL ± SD [f]	45.5 ± 10.2	68.8 ± 42.3	62.6 ± 24.4
Inflammation-related biomarkers	**CSF YKL-40, ng/mL ± SD [g]**	238.8 ± 49.2	278.8 ± 83.4	270.7 ± 69
	CSF sTREM2, ng/mL±SD (n)[h]	4.2 ± 2.3 (40)	5.3 ± 2.3 (28)	4.4 ± 1.9 (18)
	CSF PGRN, ng/mL ± SD	4.3 ± 1.2	4.2 ± 1.1	4.5 ± 1.3

* At least one APOEε4 allele
[a] Cognitively normal controls (CN) vs. DLB, prodDLB, AD and prodAD, p<0.001; prodAD vs. prodDLB and DLB, p = 0.05.
[b] CN, DLB and pDLB vs. AD and pAD, p < 0.001
[c] CN vs. AD, p < 0.001; prodDLB vs. AD, p = 0.09
[d] CN vs. DLB, prodDLB, AD and prodAD and AD vs. DLB and prodDLB, p < 0.001; AD vs. prodAD, p = 0.003
[e] CN vs. DLB, AD and prodAD, p < 0.001; CN vs. prod DLB, p = 0.006; DLB vs. AD and prodAD, p < 0.001; prod DLB vs. AD and prod AD, p < 0.001

Inflammation-related biomarkers across clinical diagnoses

Next, we analyzed differences in levels of YKL-40, sTREM2 and PGRN across groups (fig. 1, table 1). We did not find significant differences between DLB, prodDLB and CN in the levels of YKL-40 in CSF. Patients with DLB and prodDLB had lower YKL-40 levels than those with AD (both $p = 0.03$) and prodAD patients ($p = 0.006$ and $p = 0.007$, respectively). AD and prodAD had significant increased levels of YKL-40 compared with CN (both, $p < 0.01$).

	AD (n = 50)	PRODROMAL AD (n = 53)	TOTAL	P-VALUE
	74.6 ± 5.6	72.3 ± 6.3	73 ± 6.5	< 0.001
	62 (31)	60.4 (32)	57.8% (78)	0.949
	58 (29)	75 (39)	45.1 (93)	0.003
	22.5 ± 3.4	26.7 ± 2.3	25.7 ± 3.8	< 0.001
	384.7 ± 105.6	458.1 ± 72.2	583.6 ± 261	< 0.001
	694.5 ± 321	609 ± 267.7	493.8 ± 310	< 0.001
	94.1 ± 26.2	94.8 ± 39.2	76 ± 36.8	< 0.001
	295.3 ± 54.1	296.7 ± 55.7	277.8 ± 64.8	< 0.001
	4.3 ± 2.2 (36)	5 ± 2.4 (41)	4.6 ± 2.3 (163)	0.038
	4.4 ± 1.3	4.6 ± 1.2	4.4 ± 1.2	0.653

[f] CN vs. AD and prodAD, p < 0.001; CN vs. DLB, p = 0.016, CN vs. prodDLB, p = 0.06; DLB and prodDLB vs. AD and prodAD, p < 0.001

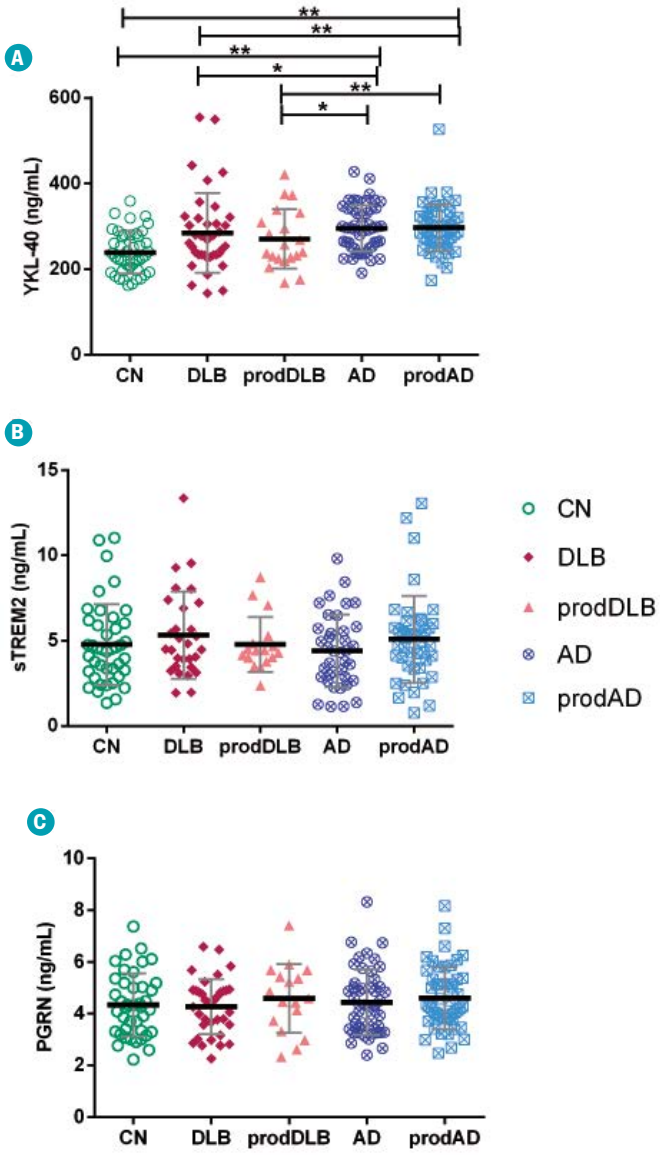
[g] CN vs. AD and prodAD, p <0.01; DLB and prodDLB vs. AD, p = 0.03; DLB and prodDLB vs. prodAD, p = 0.006 and p = 0.007, respectively

[h] pAD vs. AD, p = 0.006; AD vs. DLB, p = 0.02 (results not adjusted by multiple comparisons). pAD vs AD, p = 0.06 (results adjusted by multiple comparisons)

All p-values were adjusted by Bonferroni correction for multiple comparisons.

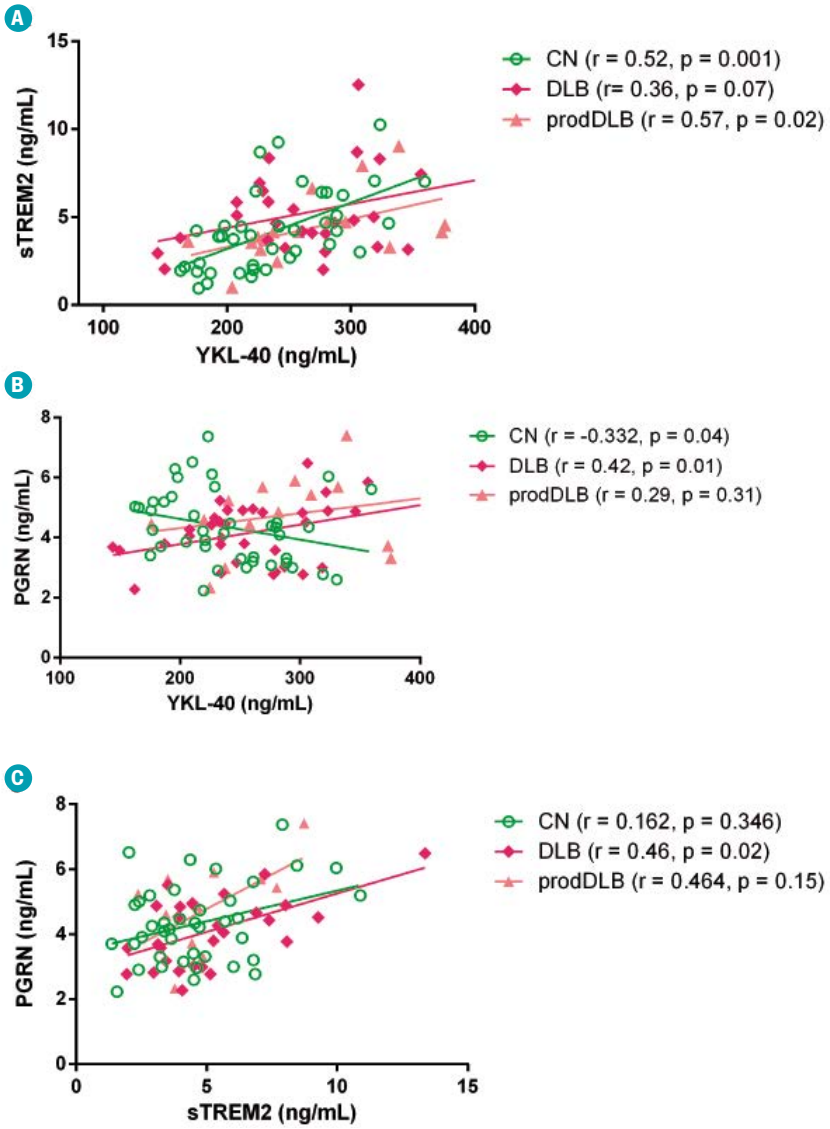
sTREM2 levels did not differ between DLB groups and CN, but they were higher in both, DLB and prodAD, compared to AD (p = 0.02 and p = 0.006, respectively). These differences, however, disappeared when adjusting by Bonferroni correction for multiple comparisons. A trend towards higher sTREM2 levels in prodAD compared to AD (p = 0.06) was observed after correcting for multiple comparisons. No differences were found across groups in CSF PGRN levels.

Figure 1. Inflammation-related biomarkers across clinical diagnoses.



*p < 0,05, **p < 0.01. CN: cognitively normal controls, DLB: Dementia with Lewy Bodies, prodDLB: prodromal DLB, AD: Alzheimer's disease, prodAD: prodromal AD. **a.** CSF YKL-40 levels in the different clinical groups. **b.** CSF sTREM2 levels in the different clinical groups. **c.** CSF PGRN levels in the different clinical groups

Figure 2. Correlations between glial biomarkers



CN: cognitively normal controls, DLB: Dementia with Lewy Bodies, prodDLB: prodromal DLB, AD: Alzheimer's disease, prodAD: prodromal AD. **a.** Correlations between CSF sTREM2 and YKL-40 levels in the different diagnostic groups. **b.** Correlations between CSF PGRN and YKL-40 levels in the different diagnostic groups. **c.** Correlations between CSF sTREM2 and PGRN levels in the different diagnostic groups

Correlations between glial biomarkers

Fig. 2 and table 2 show the correlations between the three investigated glial markers. In supplementary fig S1 we show the correlations stratified by diagnosis. We found a correlation between YKL-40 and PGRN levels in DLB ($r = 0.42$, $p < 0.05$). There was no correlation between sTREM2 and YKL-40 ($r = 0.53$, $p < 0.01$) or PGRN in DLB groups.

Table 2. Partial correlations (r values) between glial activation markers across diagnoses

	CN (n = 44)	DLB (n = 37)	PRODROMAL DLB (n = 23)	AD (n = 50)	PRODROMAL AD (n = 53)	ALL SAMPLE
YKL-40 / sTREM2, r	**0.524****	0.36	0.569[t]	**0.438***	**0.53****	**0.406*****
YKL-40 / PGRN, r	-0.332	**0.420***	0.294	0.042	**0.384***	0.171
sTREM2 / PGRN, r	0.162	-0.73	0.463	0.276	**0.499****	**0.337****
YKL-40 / Ab42, r	**0.423***	-0.73	-0.331	0.031	0.101	**-0.163***
YKL-40 / t-tau, r	0.263	**0.600*****	**0.71****	0.317	**0.65*****	**0.572*****
YKL-40 / p-tau, r	0.285	**0.627*****	**0.778*****	0.306	**0.591*****	**0.589*****
sTREM2 / Ab42, r	0.231	0.196	0.042	0.389[t]	0.409	0.152
sTREM2 / t-tau, r	**0.406***	0.235	0.459	0.078	**0.418***	**0.179***
sTREM2 / p-tau, r	**0.531****	0.272	0.455	0.155	0.368[t]	**0.204****
PGRN / ab42 r	-0.378[t]	-0.009	-0.129	-0.074	0.210	-0.108
PGRN / t-tau r	0.126	0.339	0.263	0.240	**0.393***	**0.256*****
PGRN / p-tau, r	0.075	0.415[t]	0.449	0.236	0.394	**0.294*****

[r] values in bold represent the significant correlations between biomarkers. All results have been corrected by age and multiple comparisons (Bonferroni)
* $p < 0.05$
** $p < 0.01$
*** $p < 0.001$
[t] Statistical analyses showed a tendency with a p-value between 0.09 and 0.05

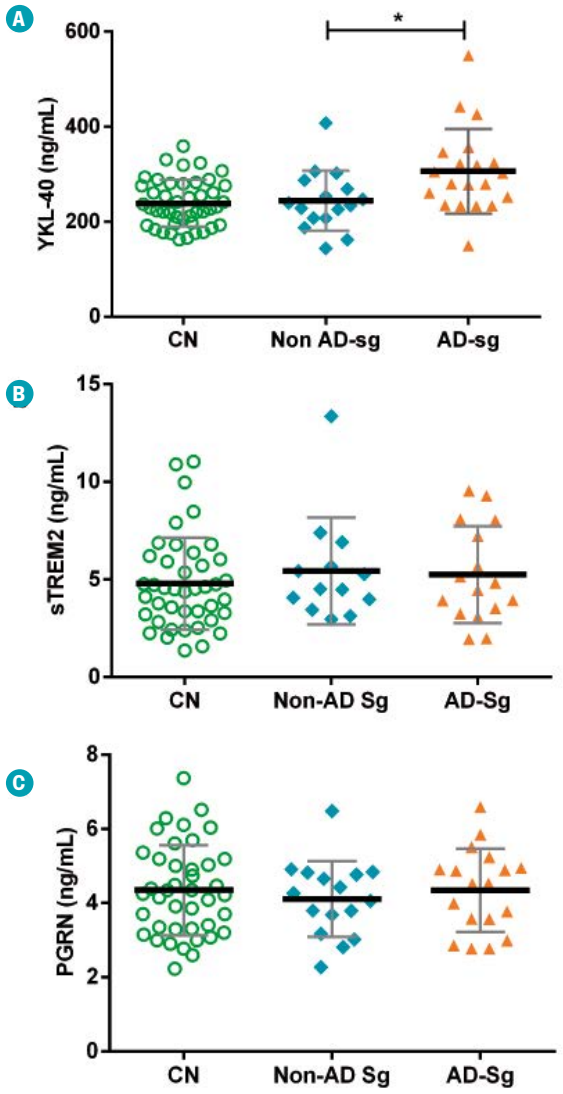
Relationship between glial and core AD biomarkers.

Table 2 and supplementary fig. S2 show the different correlations between the glial and the core AD biomarkers in CSF. YKL-40 correlated with t-tau and p-tau in prodDLB ($r = 0.71$ and $r = 0.778$, both $p < 0.001$), and DLB ($r = 0.6$ and $r = 0.627$, both $p < 0.001$). sTREM2 and PGRN levels did not correlate with Aβ42, t-tau or p-tau in the DLB groups.

Influence of AD copathology on CSF glial markers in DLB

We next analyzed levels of YKL-40, sTREM2 and PGRN in DLB patients according to the presence or absence of a CSF AD profile based on the ratio t-tau/Aβ1-42 (167). We found that levels of YKL-40 in DLB patients with a t-tau/Aβ1-42 ratio indicative of AD pathophysiology (>0.52) were higher than those with a normal t-tau/Aβ42 ratio (<0.52, fig. 3 a, $p = 0.04$). There were no differences in the CSF levels of sTREM2 and PGRN in DLB patients when stratifying by the tau/Aβ1-42 ratio (fig. 3 b and c). Suppl. table 1 shows demographic and clinical data from those patients.

Figure 3. Influence of AD copathology on CSF glial markers in DLB patients



CN: cognitively normal controls, DLB: Dementia with Lewy Bodies, Non AD-Sg: patients with core CSF biomarkers non-suggestive of concomitant AD copathology (t-tau/Aβ42 ratio <0.52). AD-Sg: patients with core CSF biomarkers suggestive of concomitant AD copathology (t-tau/Aβ42 ratio >0.52). **a.** CSF YKL-40 levels in CN, DLB patients without suspected AD copathology and DLB patients with suspected AD copathology by CSF core biomarkers. **b.** CSF sTREM2 levels in CN, DLB patients without suspected AD copathology and DLB patients with suspected AD copathology by CSF core biomarkers. **c.** CSF PGRN levels in CN, DLB patients without suspected AD copathology and DLB patients with suspected AD copathology by CSF core biomarkers.

Discussion

The main finding of this study is that YKL-40 levels are elevated in CSF in DLB patients only when there is a CSF profile suggestive of concomitant AD pathology. The glial markers YKL-40, sTREM2 and PGRN are not increased in CSF in DLB when comparing with CN. We also measured for the first time these glial markers in the prodromal phase of DLB and found no increase in this stage of the disease compared to CN and the more evolved stages in the disease.

The lack of increase in CSF YKL-40 in the whole DLB group agrees with previous findings in PD and DLB (175,176,178,179,253), supporting the absence of an increase of this protein in CSF in synucleinopathies. This may suggest a lack of astroglial activation following the α-synuclein pathology. On the other hand, our group and others have shown an increase of CSF YKL-40 in AD and also FTLD-related syndromes early in the disease course (165,167,176,177,179,245,254). This contrast between synuclein- and tau-related neurodegenerative dementias suggest that YKL-40 is particularly involved with this second group of disorders. Moreover, we demonstrate an increase in YKL-40 in DLB patients with concomitant AD copathology when compared with DLB patients without AD copathology, suggesting that it is comorbid AD what is driving astrocytic activation in DLB. In agreement with this finding, YKL-40 levels highly correlated with t-tau and p-tau levels in DLB groups. One possible explanation is that the α-synuclein inclusions observed in astrocytes in DLB may influence the astrocytic response toward neurodegeneration compared to tauopathies such as AD and FTLD (255,256).

This is the first study that reports the CSF sTREM2 levels in DLB patients. We found higher levels of this protein in DLB compared to AD, but this

difference did not survive correction for multiple testing, perhaps due to the relatively small sample size that limited the statistical power. Although TREM2 has not been previously investigated in postmortem DLB, some studies have shown higher levels of TREM2 in brains of PD patients and in PD murine models (257–259). This could support the hypothesis that TREM2 is elevated in synucleinopathies in contrast to YKL-40. Nevertheless, we could not find differences in the sTREM2 levels between DLB and CN. We did not find differences either when stratifying the DLB group by CSF AD profile, indicating that the levels of sTREM2 in DLB are independent of AD copathology. In agreement with this finding, we did not find a correlation between sTREM2 and t-tau and p-tau in DLB groups. Taken together our findings suggest a different pattern of glial activation in DLB compared to AD.

Finally, as previously reported in a smaller study (179)[29] we did not find any difference in the levels of PGRN protein in CSF in DLB and no influence of the concomitant AD pathology was detected.

One of the strengths of this study is that we included a group of patients with prodromal DLB. Although there are no established criteria for this stage of the disease, we included only those patients that converted to DLB during the follow-up. YKL-40, sTREM2 and PGRN levels have never been investigated in CSF in prodromal DLB, nevertheless, we did not detect any increase in this stage, indicating that these glial markers do not change significantly early in the disease course. Nonetheless, it is of value to include prodromal DLB patients in biomarker studies, not only to find markers of early disease stage, but also to generate new hypothesis regarding the physiopathology of the disease. On the other hand, this study has some limitations: the study is based on CSF biomarkers in a single-center cohort and needs validation in a larger independent cohort, the sample size is relatively small, relied

on clinical diagnosis and neuropathological confirmation was not available. In summary, we report that DLB and AD show different patterns of glial activation markers in CSF. YKL-40 is only increased in DLB when there is underlying AD pathology and, in contrast to AD, YKL-40 levels are not elevated in prodromal stages. We could not find differences between DLB and healthy subjects in CSF sTREM2 or PGRN levels, although a trend for higher sTREM2 levels was found compared to AD and independently on AD biomarkers. Together, these results suggest a different pattern of glial activation between DLB and AD, which needs further functional and molecular studies to elucidate the differential role of this innate immune response in DLB and its impact in the disease pathogenesis and evolution course.

Methods

Study participants and clinical classification

We prospectively included 207 subjects evaluated at the Memory Unit at Hospital de Sant Pau between January 2009 and October 2017. Patients had the following diagnoses: DLB (n = 37), prodromal DLB (prodDLB, n = 23), AD dementia (AD, n = 50) and prodromal AD (prodAD, n = 53). We also included 44 cognitively normal controls (CN) selected from the Sant Pau Initiative on Neurodegeneration (SPIN) cohort (https://santpaumemoryunit.com/our-research/spin-cohort/). To minimize the effect of gender and age, AD and prodAD cases were age- and gender-matched with the DLB and prodDLB cases. All participants received a clinical and formal neuropsychological assessment and underwent lumbar puncture to obtain CSF as reported elsewhere (167,190). DLB patients were evaluated using a previously reported clinical protocol (203).

Patients with DLB met consensus criteria for probable DLB (13). Patients with prodDLB met general criteria for MCI (108) with at least one sign

of α-sinucleinopathy (visual hallucinations, parkinsonism, or REM sleep behaviour disorder (RBD)) (25,115) and had to meet criteria of probable DLB (13) during the follow up. DLB and prodDLB patients with suspected AD copathology were defined according to the ratio t-tau/Aβ1-42 (≥0.52; indicative of underlying AD pathology) (167). Patients with AD dementia and prodAD met the NIA-AA criteria (188,260,261) and all had a CSF AD profile defined by low Aβ1-42 and high tau or p-tau levels according to our published cut-offs (167).

CN were volunteers with a normal neuropsychological evaluation for age and education, normal levels of core AD biomarkers in CSF, and no cognitive complaints.

CSF collection and analyses

CSF was obtained by lumbar puncture as described (167). Levels of core AD biomarkers (Aβ1-42, total tau, and phosphorylated tau), YKL-40 and PGRN in CSF were measured using commercially available kits from Fujirebio-Europe (InnotestTM), Quidel and Adipogen, Inc., respectively, as previously described (167,179). sTREM2 levels were measured by ELISA using previously described methods (169,172,262). All samples were randomized across plates, measured in duplicate, and all included samples had an intraplate coefficient of variation (CV) <15%. The operator was blinded to clinical diagnosis as in previous studies (169,172).

APOE genotyping

DNA was extracted using standard procedures and APOE was genotyped accordingly to previously described methods (263).

Statistical analysis

Differences in categorical variables were assessed by Pearson chi-square

tests. Normality of the variables was tested by Shapiro-Wilk test. Non-normally distributed variables (sTREM2, YKL-40, total tau, and phosphorylated tau) were log10-transformed to achieve a normal distribution all the analyses with these variables were performed with the log-transformed values. Aβ1-42 did not follow a normal distribution even after log-transformation and, therefore, non-parametric tests were used. Group comparisons between normally distributed values were performed by an analysis of covariance (ANCOVA) adjusting by the effect of age. CSF sTREM2 comparisons were additionally adjusted by the effect of gender. Partial Pearson Product-Moment correlations controlled by age (and gender in CSF sTREM2) were used to test the association between continuous variables. Aβ1-42 differences between groups were tested by Kruskal-Wallis and Mann-Whitney tests. Non-parametric correlations (Spearman) were used with variables that did not follow normal distribution (Minimental State Examination (MMSE)). Bonferroni posthoc correction was applied to adjust for multiple comparisons. The level of significance was set at 5% ($\alpha = 0.05$). All statistical analyses were performed using SPSS software version 21.0 for Windows.

Ethical Approval and Consent to participate

All subjects signed the informed consent form to participate in the study and all study protocols were approved by the local ethics committee at Hospital Sant Pau.in accordance to Declaration of Helsinki.

Author´s contributions

Research project: Conception: E. M-R, A. L. Organization: E M-R., A.L. Execution: E. M-R, M. S-C. D. A., L. M-Ll
Statistical Analysis: Design: E. M-R, A.L. Execution: E M-R. Review and Critique: All authors

Manuscript: Writing of the first draft: E M-R, J. F., A. L. Review and Critique: All Authors.
All authors read and approved the final manuscript.

Competing interest

The authors declare that they have no competing interests

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Availability of supporting data

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request

SUPPLEMENTARY MATERIAL

Tittle: Different pattern of CSF glial markers between dementia with Lewy bodies and Alzheimer's disease

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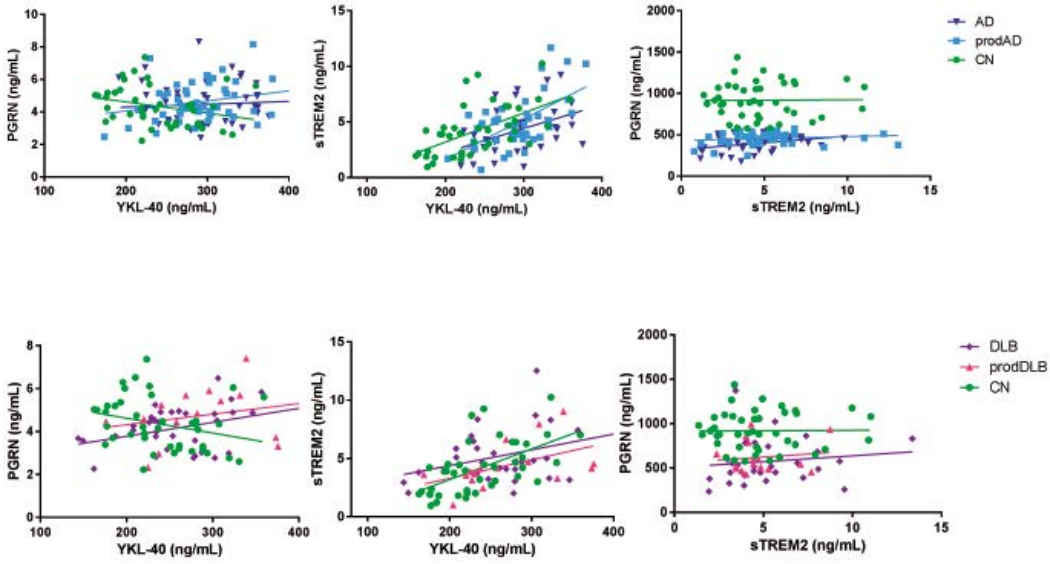
Suppl. table S1. Demographic and basic clinical data from DLB patients

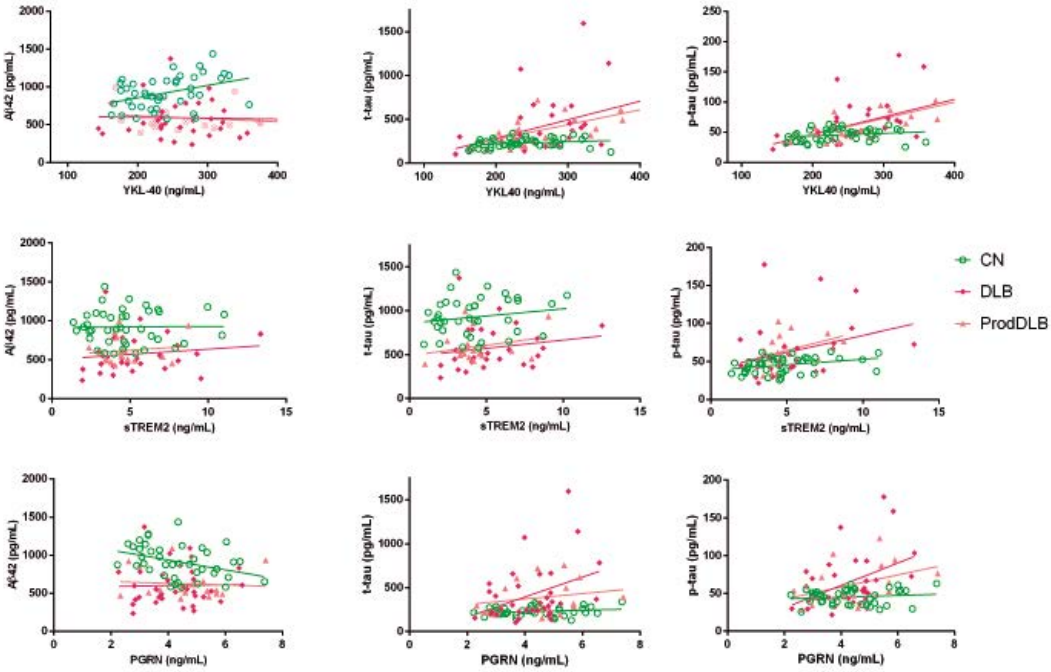
	DLB WITH AD COPATHOLOGY (n = 20)	DLB WITHOUT AD COPATHOLOGY (n = 17)	P-VALUE
Age, y ± SD	77.1 ± 1.1	75.7 ± 1.2	0.420
Sex, Females % (n)	55% (11)	52.9% (9)	1
MMSE, mean ± SD	21.9 ± 1.2	24.2 ± 0.8	0.165
Mean follow-up time (y) ± SD	2.2 ± 0.5	2.6 ± 0.6	0.568
***APOEε4*, %* (n)**	30% (6)	17.6% (3)	0.462

Supplementary figure S1. Relationship between glial biomarkers by diagnosis



CN: cognitively normal controls, DLB: Dementia with Lewy Bodies, prodDLB: prodromal DLB, AD: Alzheimer's disease, prodAD: prodromal AD.

Supplementary figure S2. Relationship between glial and core AD biomarkers



CN: cognitively normal controls, DLB: Dementia with Lewy Bodies, prodDLB: prodromal DLB,

SUPPLEMENTARY METHODS

Supplementary table S2. Intra and inter-assay CV% per measured protein.

ASSAY	INTRA-ASSAY CV%*	INTER-ASSAY CV%**
Aβ1-42	2.01%	75.7 ± 1.2
t-tau	2.01%	10.89%
t-tau	1.77%	17.24%
sTREM2	3.9%	13.02%
YKL-40	4.03%	6.3%
PGRN	4.47%	10.7%

* Intra-assay CV% was calculated as the mean of all CV% from the samples included per assay
** We included the following internal controls per assay to calculate the inter-assay CV%. Internal controls are samples with an already known concentration that we included in all the assays. In Braquets there is the mean from all assays and the interplate CV % of each internal control. Aβ1-42: L10-001 (mean: 392.5 pg/mL, CV%: 10.85%), Aβ384 (mean: 359 pg/mL, CV%: 12.48%), Aβ500 (mean: 472,73 pg/mL, CV%: 9.87%). t-tau: tau300 (mean: 297.78 pg/mL, CV%: 10.89%). p-tau: pTau125 (mean: 132.98 pg/mL, CV%: 17.24%). sTREM2: Interplate D (mean: 3.08 ng/mL, CV%: 11.54%) and Interplate E (mean: 4.44 ng/mL, CV%: 14.51%). YKL-40: YKL40H (mean: 142.84 ng/mL, CV%: 6.3%). PGRN: L10-84 (mean: 3.67 ng/mL, CV%: 15.45%) and L15-033 (mean: 5.12 ng/mL, CV%: 5.12%)

Standard curve calculation per assay

- Standard curves for Innotest assays (Aβ1-42, t-tau and p-tau) were calculated with a 4-parameter logistic regression (provided by the manufacturer).
- Standard curves for sTREM2 assays were calculated by with a 4-parameter logistic regression in the MSD software.
- Standard curves for YKL-40 assays were calculated by a polinomic regression in Excel as recommended by the manufacturer.
- Standard curves for PGRN assays were calculated by a polinomic regression in Excel as recommended by the manufacturer.

All the assays had standard curves with a R squared over 0.95

6. CHAPTER VI: DISCUSSION

This thesis has focused on the clinical and physiopathological heterogeneity of DLB, studying the disease from different perspectives. First, I describe three different clinical subtypes according to the predominant features during the first years of the disease that show different disease evolutions: a cognitive-, a neuropsychiatric- and a parkinsonism-predominant subtype. These results underscore the importance of the prodromal phase of the disease, a stage for which we do not have yet consensus diagnostic criteria. The different clinical subtypes in this stage of DLB have important implications from the diagnostic and prognostic points of view. Also of note, the thesis describes a novel clustering method to objectively determine these clinical subtypes based on estimation of the phenotypical contribution of hallucinations and parkinsonism in the prodromal stage. This thesis also builds on the heterogeneity of the frequent sleep disturbances in DLB and confirms the need for V-PSG to correctly diagnose the sleep problems associated with DLB (chapter IV). That chapter describes in detail the clinical and polysomnographic characteristics of sleep in a cohort of mild DLB and emphasizes the importance of V- PSG to correctly diagnose RBD, a core diagnostic feature in DLB. A variety of conditions may mimic RBD, consequently it often goes unnoticed when using a clinical approach. Misdiagnosis of sleep disturbances may obscure the diagnosis of DLB and lead to unsuitable treatments. This thesis also describes for the first time the correlation between the awake occipital EEG frequency and the rate of EMG activity in REM sleep and proposes a new and more descriptive method to study sleep in neurodegenerative diseases, as the frequent sleep structure abnormalitites present in these disorders may hinder the application of the standard method.

In addition, this thesis studies the physiopathological heterogeneity of the glial contribution to the disease and in the context of AD copathology. I

show that CSF levels of glial markers were equivalent to controls in DLB and in prodromal DLB. Furthermore, YKL-40 was elevated only in DLB patients with AD copathology (classified according to core AD CSF biomarker levels). CSF YKL-40 also correlated with t-tau and p-tau in DLB and prodromal DLB further supporting its relationship to AD copathology. In contrast, neither sTREM2 nor PGRN correlated with any of the AD core biomarkers in either DLB or prodromal DLB. Based on these findings, I describe a differential pattern of glial activation between DLB and AD and their prodromal stages.

Across the following sections, I discuss each main issue addressed in chapters III, IV and V

6.1. The clinical heterogeneity in DLB and its significance

6.1.1. Clinical Subtypes in DLB

The existence of clinical subtypes within the DLB syndrome has been previously suggested (94). Nevertheless, rather than systematic studies specifically aimed at disentangling the heterogeneity of the disease, these studies were largely based on a review of the previously reported phenomenology. Most of the previously suggested subtypes have focused on the presence or absence of psychiatric manifestations and their features, and thus they are used to differentiate between cognitive subtypes and psychotic and/or delirium subtypes. Chapter III also describes a cognitive-predominant and a neuropsychiatric-predominant subtype, but we did not find a subtype specifically based on delirium present at onset, as those patients were extremely rare in our cohort. This difference with previous publications might be due to a publication bias, with a higher likelihood of reporting the most severe or striking cases that required hospitalization or institution-

alisation. The focus on the presence of hallucinations or psychiatric manifestations for the diagnosis of DLB may result in misdiagnosis such that patients with dementia and hallucinations are most often misdiagnosed as DLB but found to have a diagnosis of AD upon neuropathological examination (264). A motor predominant subtype has not been proposed before within DLB heterogeneity, despite the frequent occurrence of motor symptoms at DLB onset (18).

In contrast to DLB, clinical heterogeneity in PD has been more profoundly studied. In a similar way to the clinical subtypes of DLB described in this thesis, clinical subtypes that differ in their age-at-onset and progression rate have been also proposed in PD (192–194,265). Although a partial overlap exists, there are important differences between the subtypes described in PD and the DLB subtypes presented in chapter III. In particular, late onset PD typically shows a faster clinical decline and more frequent co-occurrence of cognitive symptoms (192–194,265), whereas in DLB, the neuropsychiatric-predominant subtype described here is characterized by an older age of onset, but not by a more rapid evolution. In addition, the cognitive-predominant DLB subtype characterized by the predominance of cognitive symptoms from onset, shows the most benign evolution. Of note, our parkinsonism-predominant subtype is characterized by a faster evolution compared with the other two subtypes on top of the predominance of motor symptoms during the prodromal phase of the disease. Considering the age of onset and the evolution, there is an overlap between the late onset PD subgroup and the parkinsonism-predominant DLB subtype described in this thesis. On the other hand, the cognitive- and neuropsychiatric-predominant subtypes described in chapter III differ substantially from the clinical subtypes previously reported in PD.

Data driven analysis for unbiased classification of the clinical heterogeneity in DLB

One of the novelties of this thesis is that subtypes were obtained by a data-driven method. Data-driven methods allow researchers to study the clinical heterogeneity as well as other aspects of neurodegenerative diseases, avoiding possible biases in defining time points or other artificial criteria to classify patients. Different clustering methods have been previously applied in the study of different neurodegenerative diseases including the clinical heterogeneity in PD (91,92,191–194). Nevertheless, the clinical heterogeneity within the DLB spectrum has not been specifically studied by clustering tools until now. In our study, we focused on the clinical features present during the prodromal phase of the disease since cluster analyses depend highly on the selection of variables.

6.1.2. The heterogeneity of sleep disturbances in DLB

Chapter IV includes a comprehensive examination of the variety of sleep disturbances present in DLB using V-PSG. In agreement with previous studies (129,130), the chapter stresses the importance of performing V-PSG to correctly diagnose and study sleep problems in DLB patients, when compared to clinical history alone. To understand the importance of V-PSG in the study and diagnosis of sleep disturbances in DLB we should consider the heterogeneity of their etiology. Many comorbidities and medications used to treat DLB patients can contribute to sleep alterations in addition to the degeneration of brain structures related to sleep, generating a variety of sleep disturbances. Clinically indistinguishable, the distinct causes of sleep problems in DLB can only be correctly diagnosed by V-PSG. Moreover, many of these causes correspond to treatable conditions, which can be missed if a V-PSG is not performed. This may lead to incorrect treatment, which can

have a negative impact not only on the quality of sleep, but also on the cognitive performance, fluctuations and neuropsychiatric symptoms. The worsening of this symptomatology negatively affects the patient's quality of life and increases the caregiver overload. Thus, the appropriate diagnosis and subsequent treatment of sleep disturbances in DLB is crucial for the correct clinical management of the disease.

RBD in DLB

Another key aspect of chapter IV is the thorough study of RBD in DLB from a clinical and electrophysiological point of view. RBD is a core diagnostic feature in the new consensus diagnostic criteria for DLB (13). Therefore, its identification is important not only from a clinical perspective but also from a diagnostic point of view. RBD is indicative of the existence of an underlying synucleinopathy (DLB, PD or MSA), so much so that that the mere presence of polysomnographic evidence of RBD in a demented patient without other symptoms is sufficient to diagnose DLB (13).

However, not all DLB patients suffer RBD. RBD was present in 50% of the DLB cohort investigated in chapter IV. Previous studies using different methodologies have reported frequencies ranging from 38% to 100% (126,128,129). These data reinforce the need for a correct diagnosis of sleep problems by V-PSG since the clinical identification of RBD is not only challenging but may also lead to a misdiagnosis of DLB. From a clinical standpoint, 46% of the patients with RBD in the studied cohort did not report unpleasant dreams and 69% were not aware of displaying RBD-related sleep behaviours when specifically asked about sleep disturbances. Thus, recognition of the syndrome in a clinical setting can be challenging, highlighting the need for a reliable sleep partner that can report the symptoms, which is not always the case. In our study, even when using the MSQ to specifically assess the presence of RBD, RBD was indistinguishable

from other conditions. In contrast, many patients who had scores suggestive of RBD in this questionnaire had other sleep disorders demonstrated by V-PSG, while other patients unaware of displaying abnormal behaviours during sleep, nor recalling nightmares had RBD confirmed by V-PSG.

This thesis also reports the correlation between the awake occipital EEG frequency and the rate of EMG activity in REM sleep in DLB patients and indicates that DLB patients with slow (<8Hz) occipital EEG activity during wakefulness were more prone to RBD. This finding might have a clinical impact as it may help to identify subjects with an increased risk of RBD with a simple EEG during wakefulness. Thus, it may be a useful biomarker in the clinical assessment of suspected DLB patients. Those DLB patients with slow occipital EEG activity may be good candidates to undergo a more detailed sleep study including a complete V-PSG. The association between RBD and slow EEG activity during wakefulness has been already described in subjects with isolated RBD who later developed mild cognitive impairment and PD (232,233). This association may be explained by impairment of the subcortical cholinergic areas. These areas, mainly the nucleus basalis of Meynert, in the basal forebrain, and the pedunculopontine/laterodorsal tegmental nucleus in the brainstem are close to the brainstem areas generating REM sleep (234). These nuclei are damaged in patients with isolated RBD (202,235), isolated RBD that develop cognitive impairment, PD (236,237) and DLB (238). It is also known that there is a prominent reduction in acetylcholinesterase activity within the occipital cortex in DLB (227) and, in addition, anticholinesterase inhibitors can increase the frequency of the EEG during wakefulness in AD (240). These findings may relate to the presence of RBD with a more cognitive phenotype in DLB and point to the need for a holistic treatment in DLB; rivastigmine can improve RBD symptomatology and correct treatment of sleep disturbances stabilizes the cognitive and neuropsychiatric symptoms. We did not find a higher

frequency of clinically suspected RBD in the cognitive-predominant clinical subtype presented in chapter III, its presence being comparable in all subgroups. Nevertheless, the study described in chapter III was limited to clinically suspected RBD as only a limited number of patients underwent V-PSG.

The sleep-wake abnormalities in DLB challenge the study of sleep in this disease

Damage to the cholinergic nuclei in the forebrain affects the function of the reticular nucleus of the thalamus that receives the cholinergic projections from the brainstem during the normal N2 stage originating sleep spindles (226). Several alterations to the sleep-wake architecture in DLB are described in chapter IV. Among them, spindles of low frequency, as well as a reduction or absence of them, may represent a marker of cholinergic thalamo-cortical dysfunction in DLB and may also be associated with more cognitive-predominant phenotypes. The reduction or absence of sleep spindles hinders the identification of NREM sleep. Other sleep-wake abnormalities described in this thesis, such as slow occipital rhythm during wakefulness, also make differentiating the start of sleep difficult. All of this challenged the standard DLB sleep scoring method that was originally designed for non-neurologically impaired subjects. The implementation of a more descriptive system to study sleep by V-PSG in demented patients and, specifically, DLB patients is strongly recommended as it may have a clinical impact on the diagnosis.

6.2. The neurobiology underlying the clinical heterogeneity in DLB

The correlation between different phenotypes and the underlying neuropathology in DLB have been explored in different studies. Some of them showed that different regional burden of α-synuclein pathology can result in differences in clinical features at onset (64,93,183), features that are

in agreement with the clinical subtypes we reported in chapter III. Different patterns of Lewy body-related pathology have also been proposed to underlie the syndromic variety in demented patients with both Lewy and AD pathology (93). For example, a higher disruption of brainstem integrity may cause more severe motor features or sleep disturbances, while a predominant cortical distribution of Lewy body pathology may lead to a predominant cognitive syndrome (93,183). In fact, in PD, a link between a higher burden of cortical Lewy bodies and cognitive impairment as well as predominantly non-tremoric motor manifestations have been reported (97,194). Those studies support the results presented in this thesis regarding the cognitive-predominant and a parkinsonian-predominant DLB subtype as well as the great variety of sleep disturbances with different etiologic origins described in chapter III and IV, respectively.

Different α-synuclein strains that underlie differential seeding capacities inducing strain-specific pathology have been also postulated as a possible source of clinical heterogeneity. This strain-specific differential pathology may contribute to the heterogeneity within synucleinopathies leading to specific neurotoxic phenotypes (60,103).

On the other hand, the common presence of coincident pathologies such as AD (93,199) could also influence the clinical manifestations of DLB. One study showed that DLB patients with concomitant AD copathology, based on core AD CSF biomarkers, had more severe clinical manifestations, more likely visual hallucinations and a higher risk of institutionalization and death (48). The higher presence of visual hallucinations is a feature of our neuropsychiatric-predominant clinical subtype that could correspond to a subgroup within our DLB cohort with a higher concomitant AD pathology. This is supported by the fact that this group had the highest age at onset. Nevertheless, AD copathology also shortens the interval between the be-

ginning of motor symptomatology and the onset of dementia in PD, and is related to a faster progression in both PD and DLB (48,49,97,185,197). Tau copathology has also been associated with higher cortical α-synuclein deposits and is distributed in different brain areas in DLB patients when compared to AD suggesting a synergistic effect between both proteins in DLB that influences the clinical phenotype (52,64,65). Clinically, tau copathology correlated inversely with the performance in the antemortem cognitive tests in DLB patients (65). Thus, we speculate that different patterns of tau deposition may correlate with the clinical subtypes displayed in chapter III. Further clinic-pathological studies are needed to confirm this hypothesis.
Finally, genetics may also play an important role in the clinical heterogeneity in DLB. Around 59% of the heritability of DLB is based on common genetic variants not included in the recent polygenic risk scores for PD or AD. These common variants have been suggested to influence the clinical variety of the disease and may support this clinical heterogeneity (79,86). While the number of patients included in this thesis is not sufficient for a comprehensive genetic study, these patients have been included in multicentric international studies aimed at disentangling this genetic variability in DLB, studies that are included as annexes in this thesis.

6.3. Different patterns of glial biomarkers in CSF between DLB and AD.

6.3.1. YKL-40 and astroglia in DLB

The results displayed in Chapter V show that YKL-40 is not elevated in DLB compared with controls. This is in agreement with previous findings in PD and DLB (175,176,179,253), and supports the lack of increase of this protein in CSF in synucleinopathies. Since YKL-40 has been reported as an astroglial activation marker (166), the absence of YKL-40 CSF elevation in

synucleinopathies may suggest a lack of astroglial activation due to α-synuclein pathology. However, several studies have shown that α-synuclein can be endocytosed by astrocytes and generates glial inclusions that change gene expression in astroglia towards a proinflammatory phenotype (159–161). There are, nevertheless, studies on astrogliosis in PD that show low or absent astrogliosis in brain regions with α-synuclein deposits which indicates a high heterogeneity in astroglial activation in PD (158) and supports the lack of an increase of YKL-40 in CSF in synucleinopathies such as DLB. An increase of CSF YKL-40 in AD and FTLD-related syndromes early in the disease course has been reported (165,167,176,177,179,245,254). Thus, there is a contrast in YKL-40 levels between synuclein- and tau-related neurodegenerative dementias. This suggests that astroglial activation is particularly involved in this second group of disorders. Moreover, in Chapter V we demonstrate an increase in YKL-40 levels in DLB patients with concomitant AD copathology when compared to DLB patients without AD copathology, pointing to comorbid AD as the main factor driving astrocytic activation in DLB. In agreement with this finding, YKL-40 levels correlated with t-tau and p-tau levels in DLB groups. One possible explanation is that the α-synuclein inclusions observed in astrocytes in DLB may influence the astrocytic response toward neurodegeneration compared to tauopathies, such as AD and FTLD (256,266). It is important to remark that the elevation of CSF YKL-40 in DLB is driven by patients with concomitant AD pathology with a higher burden of tau pathology. YKL-40 may be useful in clinical practice as a prognostic marker considering the synergistic effect proposed between α-synuclein and tau pathologies (66,155,267).

6.3.2. Microglial activity in DLB

CSF levels of sTREM2 levels in DLB had not been previously studied prior to the work presented in Chapter V. On the other hand, studies on large

cohorts have shown increased CSF levels of sTREM2 in AD compared with controls (169,170,172,248,268). We found higher levels of sTREM2 in DLB compared to AD, but this difference did not survive correction for multiple testing, perhaps due to the relatively small sample size that limited the statistical power. Despite the lack of postmortem studies of TREM2 protein in DLB, some authors have previously reported higher levels of TREM2 in brains of PD patients and in PD murine models (257–259). This could support the hypothesis that TREM2 is elevated in synucleinopathies. Nevertheless, we could not find differences in sTREM2 levels between DLB and controls. Neither did we find differences when stratifying the DLB group by CSF AD profile, indicating that the levels of sTREM2 in DLB are independent of AD copathology. In agreement with this result, we did not find a correlation between sTREM2 and t-tau and p-tau in DLB groups.

The absence or loss of function of TREM2 can lock microglia into a homeostatic state in murine models (150), thus suggesting that CSF levels of sTREM2 can be used as a microglial activation marker in neurodegenerative diseases (169). Although we did not find higher CSF levels of sTREM2 in DLB compared to controls, there is evidence of microglial activation in synucleinopathies, even in its initial phases (267,269), which may indicate that other markers better reflect the microglial activation in DLB. Another challenge in the study of sTREM2 as a microglial marker in DLB is the difficulty in finding significant differences between groups, which can be explained by the high intragroup variability in sTREM2 levels and, subsequently, the high number of cases per group required to demonstrate differences in protein levels. However, a postmortem study focused on microglia in AD and DLB has shown a lower density of microglia in brains of DLB compared with AD and controls, as well as a higher proportion of dystrophic microglia to all microglia (270). In addition, recent analysis of RNA levels in postmortem samples of DLB patients showed an increased

expression of proteins that reflect astrogliosis but not microgliosis (163). These controversies on the role of microglia activation in synucleinopathies reinforces the heterogeneity of this group of diseases in glial activation.
I also studied CSF levels of PGRN protein in DLB and prodromal DLB without finding any difference between groups, which agrees with our previous results in a set of DLB patients and other dementias (179). As in the case of sTREM2, PGRN is not influenced by concomitant AD, suggesting that microglial activity in DLB is independent of AD copathology in contrast to astroglial activity, measured using YKL-40.

In chapter V we have addressed the possible temporal variation of glial markers across different stages of DLB by including a group of prodromal DLB. Nevertheless, we did not find differences in the levels of the three glial markers studied in prodromal DLB when compared to controls, prodromal AD or DLB. Thus, we did not detect the dynamic change in these biomarkers that have been observed in the AD continuum (167,169,170,172,173). Glial biomarkers may be useful in the study of the physiopathological role of glial cells in synucleinopathies, and, furthermore, as the modulation of microglial activity has been proposed as a potential treatment target in synucleinopathies (181), they may be also useful in the future to monitor disease-modifying treatments for DLB.

6.4. The importance of recognising the prodromal phase of DLB

The main strength of this thesis is the emphasis on characterizing the prodromal phase of DLB. The lack of diagnostic consensus criteria for this stage is an unmet urgent need in DLB research. The study of the prodromal phase of DLB has been possible thanks to the prospective recruitment of a prospective DLB cohort, including MCI patients with features suggestive of an under-

lying synucleinopathy that have been followed up over several years until they met the diagnostic criteria for DLB. In chapter III we demonstrated the relevance of this stage for recognising the different clinical subtypes. From a physiopathologic point of view, chapter V shows a lack of elevation of glial markers in the prodromal stage that suggests a differential role of glial cells in DLB with respect to AD. In chapter IV we stated that monitoring the sleep disturbances enables the recognition of the prodromal stage of DLB as, when present, they may be present years before the diagnosis of DLB.

The three clinical subtypes described in this thesis clearly support the notion that clinical heterogeneity is greater during the prodromal, rather than dementia, stage. The clinical features present during this phase may capture better the initial topography of neuronal dysfunction and loss as well as better reflect the heterogeneity existing in DLB (117,193,194). As the disease progresses, the more severe and widespread pathology may result in a more homogeneous syndrome. This greater heterogeneity during the prodromal phase of DLB importantly challenges the homogenization of diagnostic criteria during this phase, challenging its recognition (109,110).

Another important challenge to the identification of prodromal DLB is the lack of suitable biomarkers to reflect the underlying physiopathological processes, namely the deposition of α-synuclein and formation of Lewy bodies. The need for good biomarkers applicable to the prodromal phase of DLB is emphasized by one of the main results of chapter III, the existence of a cognitive-predominant subtype that partially overlaps with prodromal AD. This cognitive-predominant subtype, characterised by the dominance of isolated cognitive impairment during the first years of the disease and a slow progression, is the most common form of prodromal DLB in our cohort. This cluster partially overlaps with the typical amnestic MCI due to AD (198), due to the difficulty in distinguish these groups without bio-

markers. In fact, previous clinicopathological studies have highlighted the importance of considering DLB in the differential diagnosis of MCI, as a subset of subjects will convert to DLB during follow-up (110). Nowadays CSF biomarkers are helpful in identifying those MCI patients with underlying AD pathology related to their clinical syndrome and thus, allowing a diagnosis of prodromal AD. However, currently this is not possible in DLB, due to the lack of biochemical markers of Lewy body related pathology. Moreover, considering the high prevalence of AD copathology in DLB, it would be possible that a prodromal DLB patient had a CSF biomarker profile suggestive of AD underlying pathology. Thus, the diagnosis of prodromal DLB is obscured by the existence of biomarkers of AD and the absence of biomarkers for Lewy body related pathology. The development of specific and sensitive CSF and/or neuroimaging biomarkers for Lewy body-related pathology in the initial stages of the disease is essential to the correct clinical management of MCI.

Definition of the prodromal phase of DLB may be crucial to distinguish DLB cases when diagnosis is uncertain (50). With that aim, chapter III provided a detailed clinical description of prodromal DLB. This has been possible because of a structured chart review and the performance of a questionnaire specifically oriented to the identification of the main clinical features in DLB and a complete neuropsychological assessment. It is important to remark that all cognitive domains, and not only memory, should be assessed in MCI patients to allow the correct recognition of prodromal DLB, as attention and visuospatial domains are typically affected while memory is often preserved. This consideration is important, especially in those patients with iRBD, an important group at risk of developing DLB. The assessment based on screening tools, such as MMSE, can result in an underdiagnosis of DLB in its prodromal stage because of its lack of sensitivity to detect the earliest phases of DLB as is the case of MCI due to AD (14,111).

Chapter IV highlighted the importance of sleep disturbances in DLB. Sleep abnormalities, such as RBD symptoms, are especially relevant in prodromal DLB since when present, may precede cognitive symptoms and the full-blown picture of DLB by several years. Chapter IV also described other EEG features, such as the slow occipital rhythm, which enables identification of patients more susceptible to RBD. This finding could be useful in MCI patients as this may be useful to select candidates for a more detailed sleep study, facilitating the recognition of RBD and other sleep disturbances in DLB.

6.5. Limitations

The main limitations common to the three studies included in this thesis are the relatively small number of included patients, the lack of pathological confirmation of the DLB diagnosis and that the entire cohort was recruited in a single center that can result in some biases, such as selection bias, etc. There is also need to validate the results in an independent cohort.
Besides these common limitations, there are other specific limitations for each sub-study. In chapter III, part of the data was collected retrospectively and, due to the low number of patients who had CSF biomarkers or brain imaging, study of these biomarkers was not feasible. The main limitations in chapter IV were that more than 50% of the patients refused to participate; some patients did not achieve enough REM sleep, there was not a reliable sleep informant in 51% of cases, some patients used medications that could modify the sleep architecture, and a control group was not included.

6.6. Future directions

Here I propose the questions that go beyond this thesis and that will need future research. To validate the three clinical subtypes of DLB a study of the underlying neuropathology is required to investigate the neuropathological dif-

ferences that may relate to the different phenotypes. Most of the neuropathological studies addressing the clinical variety in DLB centred in the presence of AD copathology (65,93). To properly understand the contribution of AD copathology and other copathologies, as TDP-43 aggregates, in these clusters, neuropathological studies aimed to quantify these copathologies in the different cortical and subcortical brain areas in DLB patients with extensive and detailed clinical reports from the stage of MCI are needed. On the other hand, a neuropathological study of DLB patients subdivided according to the proposed clinical subtypes would also allow the study of the cellular and molecular biology beyond the Lewy body related pathology and AD copathology that may underlie the clinical heterogeneity in DLB. New techniques assessing the different pointed α-synuclein strains and specific seeding capacity as well as their relationship with tau aggregation should be applied in the research of the clinical heterogeneity, not merely along synucleinopathies, but within DLB itself.

The development and application of biochemical and neuroimaging biomarkers is highly relevant to disentangle the biological basis supporting the clinical heterogeneity in DLB. These markers would be essential in the detection of the prodromal phase of DLB. The recognition of prodromal DLB is highly relevant considering future disease-modifying drugs that may be more effective in this phase of the disease. The study of DLB patients from their earlier stages would allow longitudinal studies to investigate the evolution of the biological markers together with the different clinical features. Although iRBD patients are amenable to longitudinal follow-up (107), to avoid biases in extrapolating features typical from those patients to others without RBD and that may present very different characteristics (107), such studies should not be limited to this group.

Furthermore, studies aimed at exploring the relationship between the brain structure and function by MRI and the three clinical subtypes presented in

Chapter III are needed. This study could be complemented with functional neuroimaging based on PET scan, which would also be informative of the underlying damage to neural networks underlying the proposed clinical subtypes. New neuroimaging technics as amyloid- and tau- PET could also be useful to study copathology and pathological synergies in DLB. To obtain this objective, larger cohorts with appropriate and quantified neuroimaging examinations and a complete and prospective clinical assessment from the stage of MCI, are required. Since DLB has a lower prevalence and incidence than AD, and is frequently underdiagnosed, the creation of large, sufficiently powered cohorts to perform different studies is challenging. To solve this problem, it is crucial to create and enlarge common and public repositories based on multicentric and international recruitment following the example of the Alzheimer Disease Neuroimaging Initiative (ADNI). With this aim, several initiatives such as the European DLB consortium in Europe or the Dementia with Lewy bodies Consortium in United States have started recently.

From a clinical point of view, the importance of the V-PSG in the study of the sleep disturbances in DLB has been highlighted in Chapter IV. Larger studies with a systematic V-PSG analysis aimed to establish new descriptive criteria for the assessment of sleep in demented patients, specifically in DLB, are needed. These criteria would help in the harmonization of sleep studies focused on better understanding the sleep pathology in DLB. Improved systematic and specific studies of sleep disturbances in DLB in specialized clinical centres improves the clinical management of these patients. Given the limited access and the difficulties associated with performing a V-PSG on demented patients, the search for other early markers that can identify candidates who would benefit from V-PSG would be of help in clinical practice. How the different sleep pathologies in DLB correlate with specific phenotypes, biomarker profile and underlying neuropathology should also be an important point of research.

Finally, neuropathological and experimental studies using cellular and animal models and aimed at understanding the role of glial activation in DLB should be undertaken. Specifically, in vitro and in vivo studies of how glial activity influences tau pathology in synucleinopathies should be performed. Besides basic experimental studies, we should consider the importance of neuropathological studies specifically aimed to better understand the role of glial cells in the human DLB brain. Specific studies aimed at unravelling the role of glial cells in the beginning and the evolution of the DLB are needed.

7. CONCLUSIONS

- Three subtypes of DLB that differ in their clinical manifestations and progression can be distinguished based on the predominant symptoms during the prodromal phase of the disease: cognitive-, psychotic- and parkinsonism-predominant.

- Sleep-wake disturbances are common and complex in patients with DLB. V-PSG is essential to diagnose RBD and rule out mimics.

- DLB and AD show different patterns of glial activation markers in CSF. sTREM2, PRGN and YKL-40 levels in CSF are normal in prodromal DLB and DLB in the absence of AD co-pathology. YKL40 levels are only elevated in those DLB patients with abnormal core AD biomarkers.

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9. ANNEXES

Annex 1

Article: Progranulin protein levels in cerebrospinal fluid in neurodegenerative dementias

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Progranulin Protein Levels in Cerebrospinal Fluid in Primary Neurodegenerative Dementias

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Abstract.
Background: Progranulin is implicated in frontotemporal dementia (FTD), but its role in other neurodegenerative disorders is unknown.
Objective: To investigate the levels of progranulin (PGRN) in cerebrospinal fluid (CSF) in different neurodegenerative dementias and their correlation with levels in plasma in cognitively normal subjects.
Methods: We measured PGRN in CSF in 229 patients with amnestic mild cognitive impairment, Alzheimer's disease dementia, sporadic FTD, dementia with Lewy bodies, corticobasal syndrome, or progressive supranuclear palsy. We also measured PGRN in CSF and plasma in 74 cognitively normal individuals. We examined the correlation between PGRN levels in CSF and diagnosis, cortical thickness, genetic factors and other CSF biomarkers. We also investigated the correlation between plasma and CSF levels of PGRN in cognitively normal individuals.
Results: CSF levels did not differ across diagnoses or correlate with cortical thickness. Polymorphism rs5848 in *GRN* influenced CSF PGRN levels, but *APOE* ε4 allele did not. Amyloid-β_{42}, t-tau, p-tau, and YKL-40 levels correlated weakly with PGRN in CSF. We found a weak correlation ($r = 0.362$) between plasma and CSF PGRN levels in cognitively normal individuals.
Conclusions: Our findings do not support a diagnostic value of CSF PGRN in neurodegenerative diseases. Our data confirm that levels of PGRN in plasma do not reflect accurately levels in CSF in cognitively normal controls. These data should be considered in clinical trials aiming to increase PGRN.

Keywords: Alzheimer's disease, amyloid-β, biomarker, cerebrospinal fluid, dementia, plasma, progranulin, tau

INTRODUCTION

Progranulin protein (PGRN) is encoded by the *granulin* gene (*GRN*) and expressed in many tissues and cell types, where it is involved in angiogenesis, wound repair, cell proliferation, and inflammation [1–3]. In the adult brain, PGRN is expressed in neurons and microglia and it regulates neurite outgrowth and survival [4]. Heterozygous mutations in the *GRN* gene cause frontotemporal lobar degeneration with TAR-DNA-binding protein 43 inclusions (FTLD-TDP) [5–8]. Pathogenic *GRN* mutations are

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typically nonsense and splice-site mutations resulting in haploinsufficiency and low plasma PGRN levels in mutation carriers [9]. Measurement of plasma and serum PGRN levels has thus proven to be a reliable biomarker to identify symptomatic and asymptomatic carriers of *GRN* mutations [10–12].

In addition to a link between PGRN and frontotemporal dementia (FTD), findings to date suggest a relationship between PGRN expression in the brain and other neurodegenerative diseases. Functional genetic variants that modulate *GRN* expression, such as the rs5848 polymorphism, have been shown to increase the risk of Alzheimer's disease (AD) independently of the *APOE* ε4 allele [13–18]. Low brain PGRN expression has been shown to enhance amyloid-b (Aβ) and tau pathologies in transgenic mouse models of AD [19, 20]. These data suggest that PGRN may play a role in the pathophysiology of neurodegenerative dementias other than FTD.

PGRN levels can be measured in plasma and cerebrospinal fluid (CSF) but the relationship between plasma and CSF levels in subjects without *GRN* mutations has only been addressed in one study [21]. The authors found a weak correlation between plasma and CSF PGRN levels in 272 subjects, including 49 subjects with mild cognitive impairment (MCI) or AD dementia. The levels of PGRN in CSF in primary neurodegenerative dementias have not been systematically investigated. It is important to determine if PGRN is altered in the different neurodegenerative disorders as well as it relationship with other biomarkers such as cortical thickness or AD biomarkers in CSF. Finally, since PGRN is involved in the inflammatory response, it may influence other inflammatory proteins, such as the astroglial protein YKL-40 in CSF [22].

The objectives of this study were to evaluate how CSF PGRN levels differ across diagnosis in patients with primary neurodegenerative dementias without *GRN* mutations, how plasma PGRN levels correlate with CSF PGRN levels, and how these levels relate to genetic factors (rs5848 polymorphism in *GRN* gene and *APOE* ε4 allele), cortical thickness, core AD biomarkers and YKL-40 levels in CSF.

METHODS

Study participants

This study included 303 subjects attended at the Memory Unit at Hospital Sant Pau between January 2009 and October 2014. All subjects were evaluated by neurologists with expertise in neurodegenerative diseases, and all underwent formal cognitive evaluation using a previously published neuropsychological battery [23]. Participants had the following diagnoses: amnestic mild cognitive impairment (aMCI, $n=90$), AD dementia ($n=73$), FTD ($n=32$), progressive supranuclear palsy (PSP, $n=3$), corticobasal syndrome (CBS, $n=8$), and dementia with Lewy bodies (DLB, $n=23$). Patients with FTD included behavioral variant (bvFTD, $n=24$), semantic variant of primary progressive aphasia (svPPA, $n=4$), and nonfluent variant of primary progressive aphasia (nfvPPA, $n=4$). Patients with MCI and AD dementia met the recent NIA-AA criteria [24, 25], and patients with FTD met the new international consensus criteria for bvFTD [26, 27], svPPA and nfvPPA [28], PSP [29], and CBS [30]. Mutations in the *GRN* gene were excluded by direct sequencing of the coding region in FTD patients with one or more affected first-degree family member as previously described [31]. Cognitively normal controls were volunteers with a normal neuropsychological evaluation for age and education, normal levels of core AD biomarkers in CSF, and no cognitive complaints. All subjects signed the informed consent form to participate in the study, and all study protocols were approved by the local ethics committee at Hospital Sant Pau.

CSF and plasma collection and analysis

CSF was obtained by lumbar puncture as described [22, 32]. Levels of core AD biomarkers ($A\beta_{1-42}$, total tau, and phosphorylated tau) and YKL-40 in CSF were measured using commercially available kits from Fujirebio-Innogenetics (Innotest^{TM}) and Quidel, respectively, as previously described [22, 32]. Plasma was drawn on the day of the lumbar puncture in all subjects after 6 h of fasting [31]. Our laboratory has extensive experience in determining CSF biomarkers and participates in the Alzheimer's Association external quality control program for CSF biomarkers [33].

PGRN ELISA assay

PGRN levels were measured in CSF in all 303 subjects using the Human Progranulin ELISA Kit (Adipogen, Inc., Seoul, Korea) and in plasma in 74 cognitively normal subjects, as previously described [31]. Plasma and CSF samples were diluted prior to analysis at 1:200 and 1:5, respectively. Intra-and inter-assay coefficients of variation were 3.4% and 14.4% for CSF and 3.2% and 9.1% for plasma, respectively.

Magnetic resonance imaging (MRI) analysis

Routine imaging was performed in all subjects included in the study and 97 had consented to undergo a research MRI with a harmonized protocol for neurodegenerative diseases. Twelve of these 97 participants were excluded because of segmentation errors, and 85 were finally analyzed (Supplementary Table 1). There were not significant differences in age, sex, and PGRN levels in CSF between subjects with and without MRI in each clinical diagnostic group (data not shown). MRIs were acquired on a 3T MRI scanner (Philips 3.T x series Achieva). A high-resolution three-dimensional structural dataset was acquired with the following parameters: T1- weighted magnetization-prepared rapid gradient-echo, repetition time 8.1 ms, echo time 3.7 ms, 160 slices, matrix size 240 × 234; slice thickness 1 mm, voxel size 0.94 × 0.94 × 1 mm. Cortical thickness analyses were performed as previously described [34]. We studied the correlation between levels of CSF PGRN and cortical thickness. Only results that survived family-wise error correction at $p < 0.05$ were considered.

APOE and GRN genotyping

DNA was extracted using standard procedures and *APOE* was genotyped according to previously described methods [35]. Genotyping of *GRN rs5848* was performed using Taqman technology with a rs5848-specific genotyping assay (Life Technologies) and carried out in a 7900-HT Fast Real-Time PCR System (Applied Biosystems).

Statistical analyses

The normality of the variables was assessed by the Kolmogorov-Smirnov test. PGRN levels in plasma did not follow a normal distribution and were transformed on the natural logarithm (LOG) scale. Nevertheless, since results and levels of significance were similar we showed untransformed values when referring to plasma PGRN. We explored the association between PGRN levels and sex using a T-test and between PGRN levels and age using a Pearson correlation coefficient (r). We studied the association between plasma and CSF PGRN levels using a bivariate linear regression model in the group of cognitively normal controls. The relationships between clinical diagnosis, *APOE* genotype and CSF PGRN levels were explored by ANCOVA test, covariated by variables that had a significant association with PGRN levels in the single variable analyses (sex). The association between levels of PGRN and levels of $A\beta_{1-42}$, total-tau, phospho-tau, and YKL-40 in CSF was investigated by a Pearson correlation coefficient (r). The level of significance was set at 5% ($\alpha = 0.05$) and results were corrected for multiple comparisons (Bonferroni). All statistical analyses were performed using SPSS software version 21.0 for Windows.

RESULTS

Table 1 shows the demographic, clinical, and CSF biomarker data for all diagnostic groups.

Influence of sex, age and genetic factors on PGRN levels in CSF and plasma

We first investigated the influence of age, sex, and genetic factors (*APOE* ε4 and *GRN* rs5848 genotypes) on PGRN levels in CSF ($n = 303$) and plasma ($n = 74$). We did not find any correlation between age and PGRN levels in CSF ($p = 0.235$) or plasma ($p = 0.701$). Males showed higher levels of PGRN in CSF than females (4.99 and 4.66 ng/ml respectively, $p = 0.019$). How-

Table 1
Demographic and CSF data from subjects included in the study

Diagnosis	Controls ($n = 74$)	aMCI ($n = 90$)	AD dementia ($n = 73$)	FTD ($n = 32$)	PSP + CBS ($n = 11$)	DLB ($n = 23$)
Age, years	59 (8.3)	67.9 (8.7)	70 (8.4)	64.6 (9.1)	71.2	76.6 (5.19)
Sex (% men)	31.1	45.6	38.4	65.6	27.3	60.9
MMSE (SD)	29.1 (1.0)	27.4 (2.0)	22.0 (3.9)	23.7 (7.2)	23.1 (6.7)	25.7 (3.1)
CSF PGRN, ng/mL	4.9 (1.1)	4.8 (1.1)	4.7 (1.2)	4.7 (1.4)	5.2 (1.3)	5.0 (0.9)
CSF $A\beta_{1-42}$, pg/mL	811.9 (163.9)	638.4 (243.3)	352.0 (115.9)	604.5 (285.1)	524.6 (193.4)	613.4 (215.7)
CSF t-tau, pg/mL	202.1 (68.2)	389.1 (235.8)	740.0 (401.3)	313.4 (210.5)	244.8 (112.6)	356.5 (294.6)
CSF p-tau, pg/mL	40.4 (11.5)	65.5 (30.0)	91.3 (33.4)	46.3 (24.9)	41.2 (15.0)	60.5 (31.7)
CSF YKL-40, ng/mL	195.1 (44.6)	251.1 (49.8)	267.5 (52.8)	257.7 (62.3)	273.8 (57.5)	255.9 (57.6)
*APOE*ε4 (%)*	24.3	43.8	60.3	34.4	27.3	26.1
rs5848 TT (%)	4.1	10	5.5	12.5	0	4.3

Values are presented as mean (standard deviation) unless otherwise specified. *At least one *APOE*ε4 allele.

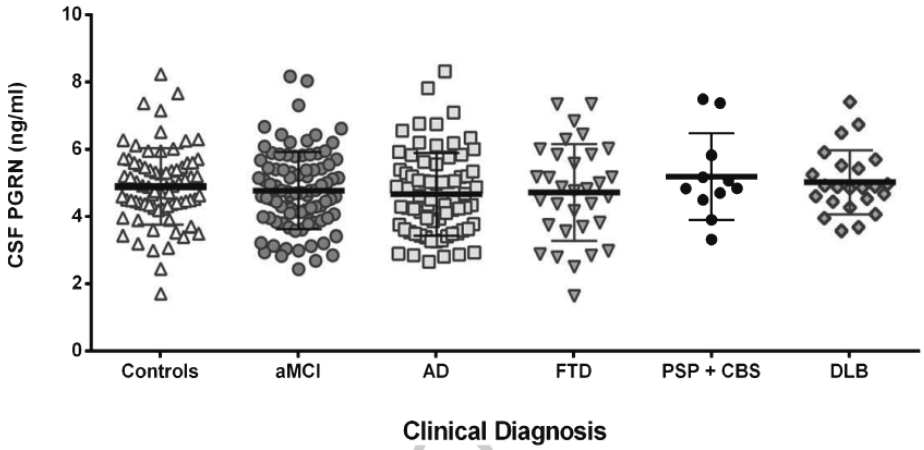

Fig. 1. PGRN levels in CSF across the different clinical diagnoses.

ever, these differences were not significant in plasma. *APOE* ε4 did not influence PGRN levels in CSF or plasma ($p = 0.585$ and $p = 0.438$, respectively). PGRN levels in CSF were influenced by the *GRN* rs5848 genotype. Subjects with the TT genotype had lower CSF PGRN levels than the group with CC and CT genotypes (3.94 and 4.87 ng/ml, respectively, $p < 0.001$). No significant effects of the rs5848 genotype on plasma PGRN levels were found.

PGRN levels in CSF across diagnoses and their correlation with clinical and neuroimaging variables

Next, we evaluated how levels of PGRN in CSF differed across a subset of common neurodegenerative diseases. Sex-adjusted analyses did not reveal any differences between groups (Fig. 1). Levels of PGRN in CSF did not correlate with MMSE scores in the whole cohort or within clinical groups. We did not find any correlation between cortical thickness and PGRN levels in CSF in the whole group of subjects with available MRI (Supplementary Table 1), or in the exploratory analysis in each clinical group.

Relationship between PGRN and other biomarkers measured in CSF

We also investigated the relationship between the PGRN protein and other AD-related biomarkers in CSF. We found a weak correlation between PGRN and $A\beta_{1-42}$ levels in CSF ($p = 0.003$, $r = 0.169$), t-tau ($p = 0.001$, $r = 0.192$), and p-tau ($p < 0.001$, $r = 0.201$) in the whole cohort (Fig. 2A–C). We also examined the relationship between PGRN with the astrocytic marker YKL-40 in CSF and found a positive correlation between the two ($p < 0.001$, $r = 0.269$, Fig. 2D) in the entire cohort.

Relationship between PGRN levels in plasma and CSF in healthy controls

To assess whether plasma PGRN can be used as a surrogate marker of PGRN in the CSF we compared PGRN levels in paired samples of plasma and CSF from 74 cognitively normal subjects in a simple regression model. The levels of PGRN in CSF were lower than those in plasma. We found a weak but significant correlation between plasma and CSF levels of PGRN ($p = 0.002$, $r = 0.362$, Fig. 3).

DISCUSSION

In this study we found that PGRN levels in CSF did not differ across a variety of primary neurodegenerative dementias. We also observed that CSF PGRN levels were largely independent of plasma PGRN levels and correlated weakly with core AD and YKL-40 biomarkers.

We first investigated the levels of PGRN in CSF across different neurodegenerative conditions without GRN mutations. The lack of differences in CSF PGRN levels between clinical groups indicates a low diagnostic value in neurodegenerative dementias. In agreement with these results, we did not find any association with MMSE scores or with cortical atrophy maps in the

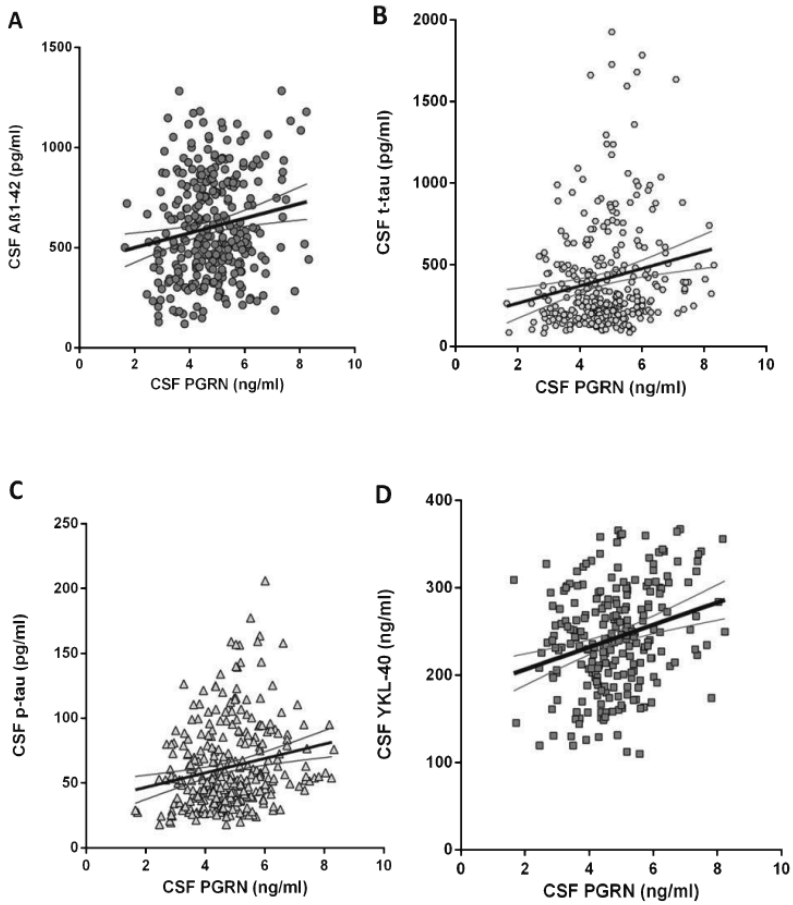

Fig. 2. A) Correlation between PGRN and $A\beta_{1-42}$ levels in CSF in the whole cohort ($n = 303$, $p = 0.003$, $r = 0.169$). B) Correlation between PGRN and t-tau levels in CSF in the whole sample ($n = 303$, $p = 0.001$, $r = 0.192$). C) Correlation between PGRN and p-tau levels in CSF in the whole sample ($n = 303$, $p < 0.001$, $r = 0.201$). D) Correlation between PGRN and YKL-40 levels in CSF in the whole sample ($n = 303$, $p < 0.001$, $r = 0.269$).

subgroup of subjects with available brain MRI. These results suggest that PGRN levels are tightly regulated in the CSF and are not disturbed by an underlying neurodegenerative process. This is in contrast with what has been observed in inflammatory disorders such as multiple sclerosis, in which elevated CSF PGRN levels have been described [36]. PGRN concentrations in CSF were also elevated in a group of patients with viral encephalitis. This suggests that an acute high-grade inflammation may be required to increase PGRN in CSF [36] in contrast with the chronic low-grade inflammation observed in neurodegenerative disorders.

Several observations to date suggest a relationship between PGRN expression in the brain and neurodegenerative diseases other than FTD. Recent studies have shown that PGRN deficiency aggravates AD pathology in animal models of AD [19, 20]. In particular, PGRN deficiency increased Aβ load and reduced phagocytosis in transgenic AβPP mouse models of AD, and accelerated tau phosphorylation and intracellular accumulation in transgenic tau mouse models [19, 20]. However, whether PGRN plays a role in the pathogenesis of patients with AD remains unclear. In this study, we found a weak correlation between PGRN levels and different AD biomarkers in CSF that may support data from transgenic animal models. However, these correlations were weak, and they did not translate into differences in PGRN levels in CSF across clinical groups with different amounts of Aβ and tau pathologies. Although the highest correla-

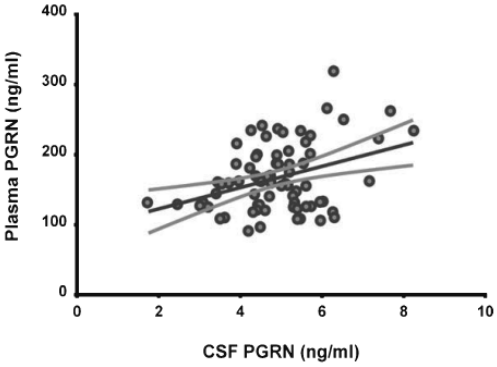

Fig. 3. Correlation between PGRN levels in plasma and in CSF in healthy subjects ($n = 74$, $p = 0.002$, $r = 0.362$).

tion was found between PGRN and the inflammatory marker YKL-40 in CSF, whether this finding reflects the known modulatory effects of PGRN on inflammation remains to be investigated.

The use of plasma PGRN levels to detect GRN mutation carriers is one of the best examples of a reliable plasma biomarker in neurodegenerative diseases [10–12]. Another example is the triggering receptor expressed on myeloid cells 2 (also known as TREM2), a protein involved in phagocytosis. Heterozygous mutations in TREM2 cause a rare FTD-like disorder syndrome [37] and plasma soluble TREM2 levels are markedly reduced in mutation carriers [38]. However, whether these "peripheral" markers are useful in common sporadic neurodegenerative conditions is uncertain. PGRN plasma levels in FTD patients without GRN mutations are typically unchanged [31]. In the case of soluble TREM2, patients with AD or FTD had plasma levels similar to those of healthy controls [38]. This raises the question of whether a "peripheral" marker can be used to monitor relevant pathophysiological changes in the CNS in common neurodegenerative diseases. To address this issue, an important step is to establish the correlation between plasma and CSF compartments. A recent study suggested that PGRN levels in plasma and CSF were weakly correlated [21]. In our study of paired samples, we observed a higher correlation (partial $r = 0.36$) between CSF and plasma PGRN levels than in the previous study (partial $r = 0.17$, [21]). However, this correlation was still weak and plasma PGRN levels explained only 13.1% of the variability of CSF PGRN levels. This is in agreement with other plasma markers investigated in neurodegenerative conditions, such as tau or YKL-40, where no or little correlation was found between plasma and CSF compartments [39, 40]. This weak correlation precludes the use of plasma PGRN as a surrogate measure of central PGRN. The permeability of the blood-brain barrier, aging and factors influencing clearance in these compartments may play an important role in influencing protein levels in each compartment. These findings have important implications for the use of biomarkers in clinical trials aiming to increase PGRN in patients with FTD. In a clinical trial setting, the measurement of PGRN could potentially be used as an indicator of target engagement of the drug. Although our study did not include subjects with *GRN* mutations, our work suggest that such a trial should include CSF measures instead of plasma for monitoring the drug treatment response. In agreement with other studies, we confirmed the influence of sex and the rs5848 in CSF PGRN levels.

The strengths of this study are the inclusion of subjects who had a detailed clinical and neuropsychological evaluation, covering several neurodegenerative dementias, and the fact that all plasma and CSF samples were drawn at the same time point. The main limitations are the lack of cases with GRN mutations, the lack of neuropathological confirmation, and the small sample size in some groups.

In summary, our results indicate that PGRN levels in CSF are tightly regulated and have no diagnostic value in primary neurodegenerative dementias not associated with *GRN* mutations. Plasma PGRN levels do not accurately reflect CSF PGRN levels in cognitively normal individuals. It would be important to investigate whether plasma PGRN levels reflect those in CSF in subjects with GRN mutations. These data are relevant for disease-modifying trials aimed at increasing PGRN.

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SUPPLEMENTARY MATERIAL

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Annex 2

Article: Investigating the genetic architecture of dementia with Lewy bodies: a two-stage genome-wide association study

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Investigating the genetic architecture of dementia with Lewy bodies: a two-stage genome-wide association study

A full list of authors and affiliations appears at the end of the article.

Summary

Background—Dementia with Lewy bodies is the second most common form of dementia in elderly people but has been overshadowed in the research field, partly because of similarities between dementia with Lewy bodies, Parkinson's disease, and Alzheimer's disease. So far, to our knowledge, no large-scale genetic study of dementia with Lewy bodies has been done. To better understand the genetic basis of dementia with Lewy bodies, we have done a genome-wide association study with the aim of identifying genetic risk factors for this disorder.

Methods—In this two-stage genome-wide association study, we collected samples from white participants of European ancestry who had been diagnosed with dementia with Lewy bodies according to established clinical or pathological criteria. In the discovery stage (with the case cohort recruited from 22 centres in ten countries and the controls derived from two publicly available database of Genotypes and Phenotypes studies [phs000404.v1.p1 and phs000982.v1.p1] in the USA), we performed genotyping and exploited the recently established Haplotype Reference Consortium panel as the basis for imputation. Pathological samples were ascertained following autopsy in each individual brain bank, whereas clinical samples were collected after participant examination. There was no specific timeframe for collection of samples. We did association analyses in all participants with dementia with Lewy bodies, and also only in participants with pathological diagnosis. In the replication stage, we performed genotyping of

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Declaration of interests
We declare no competing interests.

See Online for appendix

For the **GTEx website** see https://www.gtexportal.org/

For the **Harvard Brain Bank** website see www.brainbank.mclean.org

For the **PDGene database** see http://www.pdgene.org/

For **The Netherlands Brain Bank** see: www.brainbank.nl

For the **National Institutes of Health database of Genotypes and Phenotypes** see: http://www.ncbi.nlm.nih.gov/gap

For the full list of **contributing authors** see http://gnomad.broadinstitute.org/about

Contributors
JB, RG, JH, DJS, and AS designed the study. JB, AS, DJS, and OAR obtained funding for the study. JB, RG, OAR, CK-R, LD, SWS, and DGH did the data acquisition. JB, RG, OAR, and CK-R analysed and interpreted the data. CES, LP, SWS, OA, JC, LC, LSH, KMa, ALee, ALem, ALI, EL, ER, PStG-H, EL, HZ, IB, AB, KB, KMo, CT, SA-S, TL, JH, YC, VVD, JQT, GES, TGB, SL, DG, EM, IS, PP, PJT, LM, MO, TR, BFB, RCP, TJF, VE-P, NG-R, NJC, JCM, DJS, SP-B, DM, DWD, and GMH collected and characterised samples. JB, RG, OAR, CK-R, and TO wrote the first draft of the manuscript. All other co-authors participated in preparation of the manuscript by reading and commenting on drafts before submission.

significant and suggestive results from the discovery stage. Lastly, we did a meta-analysis of both stages under a fixed-effects model and used logistic regression to test for association in each stage.

Findings—This study included 1743 patients with dementia with Lewy bodies (1324 with pathological diagnosis) and 4454 controls (1216 patients with dementia with Lewy bodies *vs* 3791 controls in the discovery stage; 527 *vs* 663 in the replication stage). Results confirm previously reported associations: *APOE* (rs429358; odds ratio [OR] 2·40, 95% CI 2·14–2·70; $p=1·05 \times 10^{-48}$), *SNCA* (rs7681440; OR 0·73, 0·66–0·81; $p=6·39 \times 10^{-10}$), and *GBA* (rs35749011; OR 2·55, 1·88–3·46; $p=1·78 \times 10^{-9}$). They also provide some evidence for a novel candidate locus, namely *CNTN1* (rs7314908; OR 1·51, 1·27–1·79; $p=2·32 \times 10^{-6}$); further replication will be important. Additionally, we estimate the heritable component of dementia with Lewy bodies to be about 36%.

Interpretation—Despite the small sample size for a genome-wide association study, and acknowledging the potential biases from ascertaining samples from multiple locations, we present the most comprehensive and well powered genetic study in dementia with Lewy bodies so far. These data show that common genetic variability has a role in the disease.

Introduction

Dementia with Lewy bodies is the second most common form of dementia after Alzheimer's disease.[1] Despite this fact, very little attention has been devoted to understanding the pathogenesis of this disorder, particularly when compared with the other common neurodegenerative diseases such as Alzheimer's disease and Parkinson's disease.

So far, the only fully penetrant genetic variants that have been identified and replicated as a specific cause of dementia with Lewy bodies are *SNCA* point mutations and gene dosage. Three major factors might have contributed to this low number of causative mutations. First, dementia with Lewy bodies, often a disease of old age, is not commonly seen in multiplex kindreds, meaning that successful linkage studies have been rare.[2] Second, the accurate clinical diagnosis of dementia with Lewy bodies is complex, with a high rate of misdiagnosis.[3] Third, even the largest cohorts of dementia with Lewy body samples have been generally small, in many instances including as few as 100 patients.[4,5] However, the fact that dementia with Lewy bodies has a strong genetic component is currently indisputable. The ε4 allele of *APOE* is recognised to be a strong risk factor,[6,7] as are heterozygous mutations and common polymorphisms in the glucocerebrosidase gene (*GBA*).[8] Both of these results have stemmed from candidate gene association studies; *APOE* was known to be strongly associated with Alzheimer's disease and *GBA* was known to be a strong risk factor for Parkinson's disease and Lewy body disorders. In addition to these genetic associations with susceptibility, in 2016, our group provided evidence that dementia with Lewy bodies has a heritable component.[9]

No overlap in common genetic risk has been shown to exist between Parkinson's disease and Alzheimer's disease,[10] a fact that is not entirely surprising in view of the differences in phenotype. However, it is reasonable to hypothesise that the overlaps and differences in clinical and pathological presentation between dementia with Lewy bodies and both Parkinson's disease and Alzheimer's disease stem, at least in part, from aspects in their

underlying genetic architecture and, consequently, disease pathobiology. Specific genes and loci associated with disease and the strength of association are factors that can be expected to modulate these phenotypic overlaps and differences. However, despite these encouraging findings, large-scale, unbiased genetic studies of dementia with Lewy bodies have not yet been done, which is probably due to the difficulty in identifying large, homogeneous cohorts of people with the disease.

To address the need for more powerful and comprehensive genetic studies of dementia with Lewy bodies, we performed the first large-scale genome-wide association study in this disease.

Methods

Study design and participants

In this two-stage genome-wide association study, we examined data from white participants of European ancestry who had been diagnosed with dementia with Lewy bodies according to either clinical or pathological consensus criteria.[11] Most participants were diagnosed using pathological criteria and were included only when the likelihood of a diagnosis of dementia with Lewy bodies was "intermediate" or "high".[11] Samples were collected at 22 different centres across ten countries in Europe, North America, and Australia. Pathological samples were ascertained following autopsy in each individual brain bank, whereas clinical samples were collected after participant examination. There was no specific timeframe for collection of samples. White control participants in the discovery stage are part of the "general research use" controls from the two studies publicly available at the database of Genotypes and Phenotypes (The Genetic Architecture of Smoking and Smoking Cessation [phs000404.v1.p1] and Genetic Analysis of Psoriasis and Psoriatic Arthritis [phs000982.v1.p1]). For the replication stage, white controls were from the Mayo Clinic Florida control database. Investigators at every site obtained written informed consent from patients and control individuals and approval from a local ethics committee.

Discovery stage: genotyping, quality control, imputation, and statistical analysis

Participants with dementia with Lewy bodies were genotyped in either the Illumina Omni2.5M array or the Illumina OmniExpress genotyping array (Illumina, San Diego, CA, USA). Controls were genotyped in either the Illumina Omni2.5M array or the Illumina Omni1M array (Illumina, San Diego, CA, USA). Autosomal variants with GenTrain scores of more than 0·7 were included in the quality control stage. We removed single nucleotide polymorphisms (SNPs) with a call rate of less than 95%, a Hardy-Weinberg equilibrium p value in controls of less than 1×10^{-7}, or a minor allele frequency of less than 0·01. Samples were removed if they had substantial non-European admixture, were duplicates or first-degree or second-degree relatives of other samples, had a genotype call rate of less than 98%, or had substantial cryptic relatedness scores (PI_HAT >0·1).

We determined population outliers by principal components analysis, using SNPs passing the aforementioned quality-control filters. We used PLINK (version 1.9)[12] to do linkage disequilibrium-based pruning. Genotypes for remaining SNPs were combined with

1000Genomes phase 3 genotypes for samples from the YRI, CEU, JPT, and CHB reference populations, and subjected to principal components analysis. Individuals lying farther than a quarter of the distance between CEU and JPT/CHB/YRI when plotted on the axes of the first two principal components were deemed to have substantial non-European admixture and were excluded (appendix p 8).

Because samples were genotyped in a variety of arrays, we selected only variants that intersected between all arrays to be included in the imputation stage. We performed imputation using the most recent reference panels provided by the Haplotype Reference Consortium (version 1.1, 2016). We used Eagle (version 2.3) to prephase haplotypes on the basis of genotype data.[13,14] We did the imputation using the Michigan Imputation Server.[15] Following imputation, we kept variants passing a standard imputation quality threshold ($R^2 \geq 0{\cdot}3$) for further analysis.

We used logistic regression, implemented in PLINK1.9,[12] to test for association of hard-call variants with the binary case–control phenotype using sex as a covariate. We examined variants under an additive model (ie, effect of each minor allele) and estimated odds ratios (ORs) and 95% CIs. To control for population stratification, we used coordinates from the top six principal component dimensions as additional covariates in the logistic regression models. We used Q–Q plots and the genomic inflation factor (λ) to test for residual effects of population stratification not fully controlled for by the inclusion of the principal components analysis and cohort covariates in the regression model. Additionally, we have done a subanalysis in the discovery stage, including only participants with pathologically diagnosed dementia with Lewy bodies.

Moreover, to take into account the uncertainty of imputation, we have done the same association in PLINK1.9 using dosage data.

We did gene-wise burden tests using all variants with an effect in protein sequence and a maximum minor allele frequency of 5%, using SKAT-O[16,17] as implemented in EPACTS.[18] We used the top six principal components and sex as covariates in the burden test.

Replication stage: genotyping and power analysis

Replication was attempted for top variants showing a p value in the discovery stage of less than 5×10^{-6}. We tested a total of 32 signals for replication using a Sequenom MassARRAY iPLEX SNP panel (Sequenom, San Diego, CA, USA; appendix p 4). We did power calculations for replication sample size selection using the R package RPower. We estimated a mean statistical power of 81% for the 32 signals on the basis of sample size, variant frequency, and effect size in the discovery stage, and used a replication p value threshold of 0·05. We tested associations in the replication stage using logistic regression models adjusted for age (age at onset for the patients with clinically diagnosed dementia with Lewy bodies, age at death for the patients with a high pathological likelihood of dementia with Lewy bodies, and age at recruitment to study for controls) and sex.

We did a combined meta-analysis of stage 1 and 2 with GWAMA[19] under a fixed-effects model, using estimates of the allelic OR and 95% CIs.

Estimation of phenotypic variance

To estimate the phenotypic variance explained by the genotyped SNPs in this cohort, we used genetic restricted maximum likelihood analysis as implemented in the Genome-wide Complex Trait Analysis tool.[20,21] We used the first ten principal components as covariates and a disease prevalence of 0·1%.[22] We also estimated the partitioned heritability by chromosome, for which a separate genetic relationship matrix was generated for each chromosome. Each matrix was then run in a separate restricted maximum likelihood analysis. We applied linear regression to determine the relation between heritability and chromosome length.

Role of the funding source

The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

This study included a total of 1743 patients with dementia with Lewy bodies and 4454 controls. The majority of patients with dementia with Lewy bodies were neuropathologically assessed (n=1324), providing a greater level of diagnostic detail. 987 participants with dementia with Lewy bodies were genotyped with the Illumina Omni2.5M array and 700 with the Illumina OmniExpress genotyping array. 1523 controls were genotyped with the Illumina Omni2.5M array and 2847 with the Illumina Omni1M array. Application of quality control filters to the dataset at the discovery stage yielded high-quality genotypes at 448 155 SNPs for 1216 participants with dementia with Lewy bodies and 3791 controls (table 1). A total of 52 participants with dementia with Lewy bodies were excluded for cryptic relatedness, 20 for genetic ancestry, and the remaining 399 for low call rates or poor genotyping. After imputation and quality control, genotypes for 8 397 716 variants were available for downstream analyses. After linkage disequilibrium-based pruning with PLINK (version 1.9)[12] to quasi-independence (variance inflation factor=2), 130 715 SNPs remained in the dataset. The Q–Q plot and genomic inflation factor (λ=1·01) indicated good control of population stratification (appendix p 9).

Five regions were associated with dementia with Lewy bodies risk at genome-wide significance ($p<5 \times 10^{-8}$) in the discovery stage (figure 1; table 2). These regions included the previously described Alzheimer's disease and Parkinson's disease loci *APOE* (rs429358; OR 2·40, 95% CI 2·14–2·70; $p=1{\cdot}05 \times 10^{-48}$), *SNCA* (rs7681440; OR 0·73, 0·66–0·81; $p=6{\cdot}39 \times 10^{-10}$), and *GBA* (rs35749011; OR 2·55, 1·88–3·46; $p=1{\cdot}78 \times 10^{-9}$). Additionally, loci overlapping *BCL7C/STX1B* (rs897984; OR 0·74, 0·67–0·82; $p=3{\cdot}30 \times 10^{-9}$) and *GABRB3* (rs1426210; OR 1·34, 1·21–1·48; $p=2{\cdot}62 \times 10^{-8}$) were also genome-wide significant. A subanalysis including only participants with pathologically diagnosed dementia with Lewy bodies revealed that all but *GABRB3* maintained their genome-wide significance in that smaller dataset (table 2; appendix p 11). Furthermore, when undertaking the same associations in PLINK1.9 to take into account the uncertainty of imputation, results were identical to the best-guess calls (appendix p 8).

A total of 527 participants with dementia with Lewy bodies and 663 controls from the Mayo Clinic were included in the replication stage (table 3). The replication stage of the genome-wide association study design provided independent replication ($p<0{\cdot}05$) for three of the loci (*APOE*, *SNCA*, and *GBA*), all of which were also genome-wide significant in the combined analysis of both stages (table 2; appendix p 4).

In the discovery stage, suggestive evidence of an association ($p<5 \times 10^{-6}$) with dementia with Lewy bodies was also seen for two loci: *SOX17* and *CNTN1*. The association at *SOX17* did not replicate (appendix p 4). For *CNTN1*, the association with dementia with Lewy bodies (rs7314908; 1·51, 95% CI 1·27–1·79; $p=2{\cdot}32 \times 10^{-6}$) improved slightly when performing the subanalysis on the participants with pathologically confirmed dementia with Lewy bodies (rs7314908; OR 1·58, 1·32–1·88; $p=4{\cdot}32 \times 10^{-7}$), and this candidate locus showed evidence of replication with very similar effect size to that in the discovery stage (rs79329964; OR 1·54, 1·32–1·79; p=0·03; rs79329964 was used in replication as a proxy for rs7314908).

A systematic assessment of genetic loci previously associated with Alzheimer's disease or Parkinson's disease showed no evidence of other genome-wide significant associations in this dementia with Lewy bodies cohort (appendix p 5). These loci include the *TREM2* locus, where the p.Arg47His variant has been shown to have a strong effect in Alzheimer's disease.[24] In our cohort this variant did not show genome-wide significant levels of association (OR 3·46, 95% CI 1·54–7·77; p=0·002), despite the over-representation in people with dementia with Lewy bodies compared with controls. Similarly, *MAPT*, which is strongly associated with Parkinson's disease and has been previously linked to dementia with Lewy bodies,[25] shows no strong evidence of association in this study (rs17649553; OR 0·86, 0·76–0·96; p=0·0126).

To examine whether the association with *SNCA* is independent of that seen in Parkinson's disease, we conditioned our analysis on the top Parkinson's disease variant (rs356182), which showed only a negligible effect on the DLB association (conditioned OR 0·70, 95% CI 0·63–0·78; $p=2{\cdot}89 \times 10^{-10}$; figure 2). To gain insight into potential regulatory effects of this distinct *SNCA* signal, we used expression quantitative trait loci (eQTL) data from the Genotype-Tissue Expression (GTEx) Project Consortium and the Harvard Brain Bank Resource Center to determine whether rs7681440 and rs7681154 (a variant shown to have an independent association for Parkinson's disease that is in strong linkage disequilibrium [R^2=0·91] with the rs7681440 *SNCA* variant) affect gene expression as eQTLs. In the GTEx data, the most associated SNP in dementia with Lewy bodies is a strong eQTL in the cerebellum for *RP11-67M1.1*, a known antisense gene located at the 5′ end of *SNCA*, with the alternative allele showing a reduction in expression of *RP11-67M1.1* (figure 3). Additionally, rs7681154 was associated with *SNCA* expression in the cerebellum using the Harvard Brain Bank Resource Center results ($p=2{\cdot}87 \times 10^{-11}$; figure 3), with the alternative allele associated with increased *SNCA* expression.

We assessed linkage disequilibrium across the *LRRK2* locus region and that analysis revealed that rs79329964 is in equilibrium with both p.Gly2019Ser (R^2=0·000043) and with the Parkinson's disease hit at this locus, rs76904798 (R^2=0·003), suggesting rs79329964 to

be an independent association from the Parkinson's disease risk. Although samples were not screened for p.Gly2019Ser directly, the variant was well imputed (R^2=0·94). The exclusion of all samples that carried the p.Gly2019Ser variant showed no significant effect on the association at the *CNTN1* locus. Notably, the p.Gly2019Ser variant showed a higher minor allele frequency in participants with dementia with Lewy bodies (0·0021) than in controls (0·0003).

Gene-based burden analysis of all low frequency and rare variants (minor allele frequency <0·05) changing the aminoacid sequence, showed a single genome-wide significant result comprised of six variants at *GBA* (p.Asn409Ser, p.Thr408Met, p.Glu365Lys, p.Arg301His, p.Ile20Val, and p.Lys13Arg; $p=1{\cdot}29 \times 10^{-13}$). No other gene showed evidence of strong association with disease or overlapped single variant analysis results (table 4).

Using the first ten principal components as covariates and a disease prevalence of 0·1%, estimation of the phenotypic variance attributed to genetic variants showed a heritable component of dementia with Lewy bodies of 36% (SD 0·03). As expected for a common complex disease, we found a strong correlation between chromosome length and heritability ($p=6{\cdot}88 \times 10^{-5}$; figure 4).

The heritability for dementia with Lewy bodies at chromosome 19 is much higher than what would be expected considering the chromosome's size and probably reflects the role of *APOE*. Notably, chromosomes 5, 6, 7, and 13 all have higher heritability for dementia with Lewy bodies than expected, although none of them has variants with genome-wide significant results.

Discussion

This is the first comprehensive, unbiased study of common and intermediate frequency genetic variability in dementia with Lewy bodies. We identified five genome-wide significant associations in the discovery stage (*APOE*, *BCL7C*/*STX1B*, *SNCA*, *GBA*, and *GABRB3*), with the associations regarding *APOE*, *SNCA*, and *GBA* being confirmed in the replication stage and in the combined analysis of both stages.

The most significant association signal is seen at the *APOE* locus (*APOE* ε4), which has been previously shown to be highly associated with dementia with Lewy bodies.[6,7] As described, *APOE* ε4 is the major genetic risk locus for Alzheimer's disease and has been implicated in cognitive impairment within Parkinson's disease, although not with the risk of Parkinson's disease itself. The locus has also been reported to affect the levels of both β-amyloid and Lewy body pathology in brains of patients.[27] In a small Finnish dataset,[28] the ε4 allele association with dementia with Lewy bodies was largely driven by the subgroup with concomitant Alzheimer's disease pathology.

The second strongest association is seen at the *SNCA* locus. Results from our conditioned analysis confirmed the different association profile between dementia with Lewy bodies and Parkinson's disease that we had previously reported.[7] *SNCA* is the most significant common genetic risk factor for Parkinson's disease, with rs356182 having a meta-analysis p value of $1{\cdot}85 \times 10^{-82}$ (OR 1·34, 1·30–1·38) in PDGene. This variant is located 3' to the gene,[29]

whereas in dementia with Lewy bodies, no association was found in that region (figure 2). The most associated dementia with Lewy bodies SNP for the *SNCA* locus (rs7681440) has a Parkinson's disease meta-analysis p value of more than 0·05 in PDGene. When doing a conditional analysis on the top Parkinson's disease SNP (rs356182), Nalls and colleagues[29] reported an independent association at the 5′ region of the gene (rs7681154), and this variant is in strong linkage disequilibrium (R^2=0·91) with the rs7681440 *SNCA* variant identified in our study. It is tempting to speculate that these differences might reflect pathobiological differences between the two diseases, perhaps mediated by differential regulation of gene expression. The results in the GTEx data, showing that the most associated SNP in dementia with Lewy bodies is a strong eQTL in the cerebellum for *RP11-67M1.1*, are compatible with a model in which rs7681440 genotypes affect the expression levels of *SNCA* indirectly through the action of *RP11-67M1.1*. More specifically, the alternative allele associates with a decreased expression of *RP11-67M1.1* and consequently reduced repression of *SNCA* transcription (increased *SNCA* expression), which is in accordance with an increased frequency of the alternative allele in participants with dementia with Lewy bodies when compared with controls. Additionally, the relationship between rs7681154 and *SNCA* expression is supported by the high expression of *SNCA* in the brain and the association of rs7681440 with increased *SNCA* expression in whole blood ($p=2{\cdot}13 \times 10^{-38}$).[30,31] However, further investigation of the identified significant eQTLs is needed because the effect was seen for only one brain region. This localised effect could plausibly result from low overall expression of *RP11-67M1.1* and higher RNA quality in the cerebellum than in other assayed brain regions in these datasets. Notably, both eQTLs' effects fit with a model of increased *SNCA* expression in participants with dementia with Lewy bodies compared with controls.

The most significant marker at the *GBA* locus (rs35682329) is located 85 781 base pairs downstream of the gene and is in high linkage disequilibrium (D′=0·9; R^2=0·8) with p.Glu365Lys (also reported in the scientific literature as E365K, E326K, and rs2230288), which has been suggested as a risk factor for dementia with Lewy bodies.[8] The top associated variant for Parkinson's disease at this locus is the rs71628662 (PDGene meta-analysis OR 0·52 [95% CI 0·46–0·58; $p=6{\cdot}86 \times 10^{-28}$]). This variant is also in high linkage disequilibrium with the top SNP identified here (D′=0·9 and R^2=0·8). In this study, we show similar effect sizes for *APOE* (OR 2·40) and *GBA* (OR 2·55) in dementia with Lewy bodies. Gene burden-based analysis showed *GBA* as the only genome-wide significant association with dementia with Lewy bodies risk. The inexistence of other associations should be interpreted with some caution. Because we were not ascertaining the complete spectrum of genetic variability, other genes could have had a significant burden of genetic variants that were simply not captured in our study design, despite our use of the most recent imputation panel.

Although in our meta-analysis we saw a genome-wide significant association with dementia with Lewy bodies at the *BCL7C/STX1B* locus, this association was mostly driven by the discovery-stage data (replication-stage results were OR 0·98; p=0·83) and further replication is needed. That being acknowledged, an association at the *BCL7C/ STX1B* locus has been previously reported for Parkinson's disease.[29,32] The top Parkinson's disease-associated variants at this locus were rs14235 (synonymous; located at *BCKDK*) and rs4889603

(intronic; located at *SETD1A*). The top SNP identified in dementia with Lewy bodies at this locus (rs897984) shows the same direction of association seen in Parkinson's disease (OR 0·93, 95% CI 0·90–0·96), a Parkinson's disease meta-analysis p value of $1{\cdot}34 \times 10^{-5}$ (data from PDgene), and strong linkage disequilibrium with both Parkinson's disease hits (R^2=0·28–0·32; correlation p values<0·0001). This is a gene-rich region of the genome (appendix p 9), making accurate nomination of the gene driving the association difficult. Mining data from the GTEx project showed that rs897984 is not an eQTL for any gene in the locus. Nonetheless, in both Parkinson's disease studies, the nominated gene at the locus was *STX1B*, probably due to its function as a synaptic receptor.[33] Additionally, *STX1B* has a distinctive pattern of expression across tissues, presenting the highest expression in the brain. In this tissue, when compared with the closest genes in the locus (*HSD3B7, BCL7C, ZNF668, MIR4519, CTF1, FBXL19, ORAI3, SETD1A, STX4*), *STX1B* also shows the highest levels of expression (appendix p 10). In 2014, mutations in *STX1B* were shown to cause fever-associated epileptic syndromes[34] and myoclonic astatic epilepsy.[35]

Although not quite genome-wide significant in the discovery stage, the association between *CNTN1* and dementia with Lewy bodies risk replicated with a very similar association OR as the discovery stage. Interestingly, the locus has been previously associated with Parkinson's disease in a genome-wide study of identical-by-descent segments in an Ashkenazi cohort,[36] and with cerebral amyloid deposition, assessed with PET imaging in *APOE* ε4 non-carriers.[37] This locus also did not reach genome-wide significance with clinicopathological Alzheimer's disease dementia ($p=5{\cdot}21 \times 10^{-6}$).[38] The contact in 1 protein, encoded by *CNTN1*, is a glycosylphosphatidylinositol-anchored neuronal membrane protein that functions as a cell-adhesion molecule with important roles in axonal function.[39,40] Mutations in *CNTN1* were found to cause a familial form of lethal congenital myopathy.[41] Contactin 1 drives Notch-signalling activation and modulates neuroinflammation events, possibly participating in the pathogenesis of multiple sclerosis and other inflammatory disorders.[42] A functional protein association network analysis of *CNTN1* using STRING shows contactin 1 is in the same network as *PSEN2* (appendix p 11), supporting its potential role in neurodegeneration. Further replication will be important in view of the absence of a genome-wide significant association in the discovery stage; however, this association seems promising. Notably, *LRRK2* is located less than 500 000 base pairs away from the most associated SNP at this locus, which could suggest that the association might be driven by variation at the *LRRK2* locus. Further validation of the involvement of *CNTN1* variation in modifying risk of dementia with Lewy bodies will be important.

In addition to performing a genome-wide association study with clinicopathological Alzheimer's disease dementia, Beecham and colleagues[38] also analysed commonly comorbid neuropathological features seen in elderly individuals with dementia, including Lewy body disease. In this latter analysis, only the *APOE* locus was found to achieve genome-wide significance. However, when testing known common Alzheimer's disease risk variants with coincident neuropathological features, Beecham and colleagues identified hits at *SORL1* and *MEF2C*, finding them to be nominally associated. In our cohort of participants with dementia with Lewy bodies, we found no genome-wide significant associations between these variants and disease. Similarly, we had previously reported an

association at the *SCARB2* locus with dementia with Lewy bodies.[7] In the larger dataset of the present study, the association remained at the suggestive level and did not reach genome-wide significance (most significant SNP in the present study, rs13141895; $p=9{\cdot}58 \times 10^{-4}$). No other variant previously reported to be significantly associated with Alzheimer's disease or Parkinson's disease in recent genome-wide association study meta-analyses showed a genome-wide significant association with dementia with Lewy bodies. The most significant Alzheimer's disease or Parkinson's disease variants at the following loci showed nominal ($p<0{\cdot}05$) association levels: *MAPT*, *BIN1*, *GAK*, *HLA-DBQB1*, *CD2AP*, *INPP5D*, *ECHDC3*, and *SCIMP*. Additionally, variants previously suggested to be associated with Lewy-related pathology in a Finnish cohort,[28] did not show evidence of association in this study (appendix p 5). See appendix pp 12–70 for colocalisation plots of association between dementia with Lewy bodies and either Parkinson's disease or Alzheimer's disease.

This study has notable limitations. The control population is not perfectly matched to the case cohort because it was derived from publicly available data. To address this, we have used all available information (both clinical and genetic) to create a control cohort that is as similar as possible to the case cohort. Additionally, despite using the same diagnostic criteria for all included participants with dementia with Lewy bodies, diagnostic measurements were collected in a variety of locations, suggesting that diagnostic accuracy might have been variable, with contamination from participants with Parkinson's disease or Alzheimer's disease. Notably, we do not see an over-representation of genetic risk factors from those diseases in our results (eg, *MAPT*, *CLU*, or *CR1*), suggesting minimum inclusion. Similarly, population stratification could bias the results because samples were collected in various countries. In the present study, we have used standard methodology to correct for any such bias and, consequently, our results show no evidence of population stratification as evidenced by the Q–Q plot as defined by the acquired unbiased genotype data. Additionally, participants with dementia with Lewy bodies were genotyped at three locations and controls were all derived from publicly available datasets, using a mixture of genotyping arrays, which could provide a source of genotyping bias. However, our approach was to select variants that were at the intersection of all used arrays before imputation, which makes use, effectively, of the same genotyping probes for all samples. This approach has been shown to remove any bias from this type of result and any effects of using different array scanners are negligible for high-quality variants.[43]

This is the first large-scale genome-wide association study in dementia with Lewy bodies. We estimate the heritability of dementia with Lewy bodies to be approximately 36%, which is similar to what is known to occur in Parkinson's disease.[44] This finding shows that, despite not having multiple causative genes identified so far, genetics has a relevant role in the common forms of dementia with Lewy bodies. Additionally, we provide evidence suggesting that novel dementia with Lewy bodies loci are likely to be found at chromosomes 5, 6, 7, and 13 in view of the high heritability estimates at these chromosomes. A significant majority of our case cohort in the present study was comprised of participants with neuropathological diagnoses, which provide a greater level of information for diagnostic accuracy. These results provide us with the first glimpse into the molecular pathogenesis of dementia with Lewy bodies; they reveal that this disorder has a strong genetic component and suggest a unique genetic risk profile. From a molecular perspective, dementia with

Lewy bodies does not simply sit between Parkinson's disease and Alzheimer's disease; instead, the combination of risk alleles is unique, with loci that are established risk factors for those diseases having no clear role in dementia with Lewy bodies (eg, *MCCC1*, *STK39*, *CLU*, *CR1*, or *PICALM*). Further increases in the size of dementia with Lewy body cohorts will probably reveal additional common genetic risk loci, and these will, in turn, improve understanding of this disease, its commonalities, and differences with other neurodegenerative conditions, ultimately allowing the identification of disease-specific targets for future therapeutic approaches.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Research in context

Evidence before this study

We searched PubMed using the keywords "dementia Lewy bodies" AND "genetics", for manuscripts published in any language between database inception and June 21, 2017, and found no large-scale genome-wide studies of dementia with Lewy bodies. So far, most studies have focused on small cohorts and are frequently candidate gene association studies. However, in 2014, we showed that dementia with Lewy bodies has a genetic component, suggesting that a large unbiased genetic association study might provide novel loci that have a role in the disease.

Added value of this study

To our knowledge, this is the first large-scale genome-wide association study in dementia with Lewy bodies. The discovery stage included 1216 patients with dementia with Lewy bodies and 3791 controls, and the replication stage included 527 patients with the disease and 663 controls. The vast majority of people with the disease from both stages were neuropathologically diagnosed. Furthermore, despite the comparatively smaller size of the replication cohort, all samples were ascertained at the same centre, which reduces diagnostic heterogeneity.

Implications of all the available evidence

Our data show several genome-wide significant loci. Some of these loci had previously been implicated in Parkinson's disease or Alzheimer's disease, which could suggest that dementia with Lewy bodies is simply a combination of the genetic underpinnings underlying those diseases. However, our data suggest that dementia with Lewy bodies does not sit in the spectrum between Parkinson's disease and Alzheimer's disease, but instead, has a unique genetic profile. Additionally, we have also estimated the genetic heritability of dementia with Lewy bodies to be 36%, which is very close to what has been estimated for Parkinson's disease, a disease now known to have a strong genetic component.

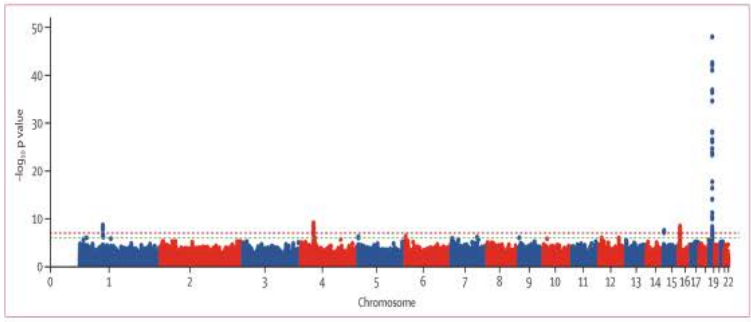

Figure 1. Manhattan plot showing genome-wide p values of association

The p values were obtained by logistic-regression analysis using the first six principal components and sex as covariates. The y axis shows $-\log_{10}$ p values of 8 397 716 single nucleotide polymorphisms, and the x axis shows their chromosomal positions. The horizontal red dotted line represents the threshold of $p=5 \times 10^{-8}$ for Bonferroni significance and the green dotted line represents the threshold of $p=5 \times 10^{-6}$ for selecting single nucleotide polymorphisms for replication.

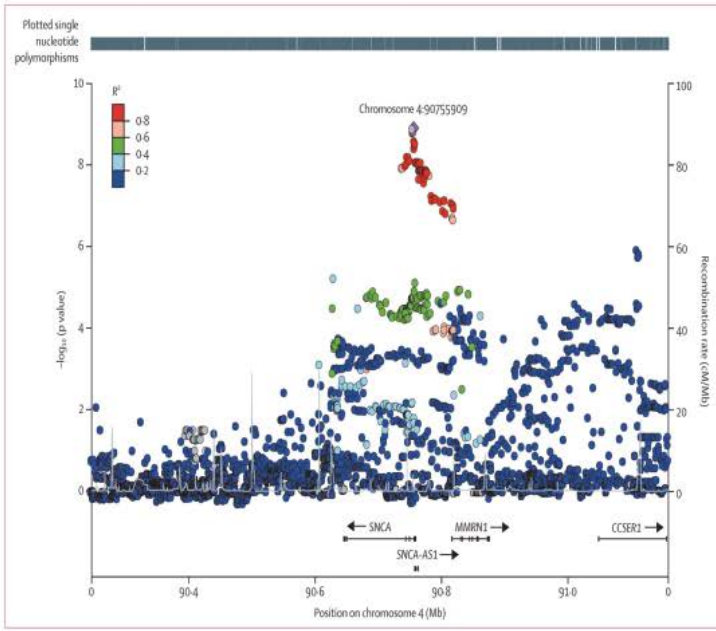

Figure 2. Regional association plot for the *SNCA* locus

Purple represents variant rs1372517, at chromosome 4, position 90755909, which is the most associated SNP at the locus also present in the 1000Genomes dataset. The variant rs1372517 is in complete linkage disequilibrium with rs7681440. Colours represent linkage disequilibrium derived from 1000Genomes between each variant and the most associated SNP. SNP=single nucleotide polymorphism. R^2 represents the level of pairwise linkage disequilibrium between the top variant and each other variant plotted, using data from the 1000 Genomes project.

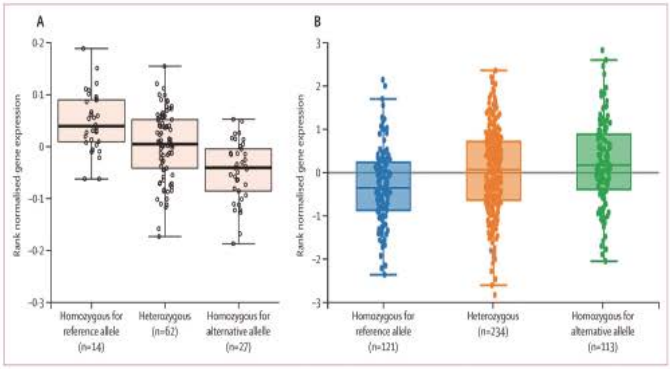

Figure 3. Associations between genotypes and gene expression in the cerebellum of post-mortem controls

(A) Association between rs7681440 genotypes and *RP11-67M1.1* expression in 103 disease-free post-mortem cerebellum samples ($p=2{\cdot}00 \times 10^{-7}$) from the Genotype-Tissue Expression (GTEx) Project Consortium. Carriers of the GG genotype (alternative allele) show the lowest levels of expression of the gene. Details on methods are on GTEx website. (B) Association between rs7681154 and *SNCA* expression ($p=2{\cdot}87 \times 10^{-11}$) in 468 disease-free cerebellum samples from postmortem individuals from the Harvard Brain Bank Resource Center.[26] Individuals with the alternative allele C had increased *SNCA* expression in the cerebellum, on average, compared with individuals with the reference allele G. Details on the subjects, experiments, and analytical methods of the expression quantitative trait loci study of the Harvard Brain Bank Resource Center samples are described by Zhang and colleagues[26] and on the Harvard Brain Bank website. Boxplots for both panels show medians, IQRs, and individual data points.

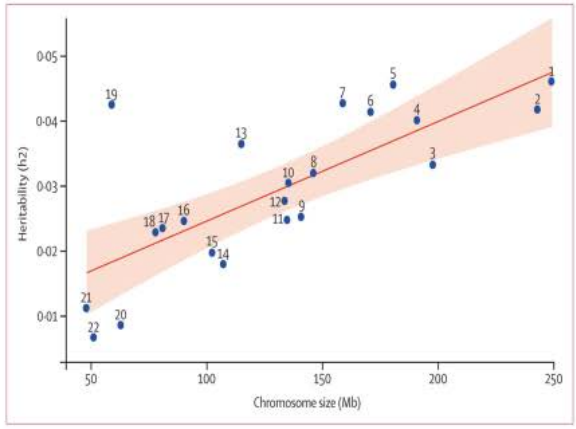

Figure 4. Dementia with Lewy bodies heritability by chromosome

Heritability (y axis) per chromosome is plotted against chromosome length (x axis). The red line represents heritability regressed on chromosome length and the shaded area represents the 95% CI of the regression model.

Table 1

Characteristics of the discovery cohort

	N	Neuropathological diagnosis		Men:women ratio	Age at onset (years)	Passed quality controls	
						Total	Neuropathological
Patients with dementia with Lewy bodies							
Australia	79	79 (100%)		1·93	65·2 (10·3)	72 (91%)	72 (91%)
Canada	29	15 (52%)		2·22	67·9 (7·8)	6 (21%)	3 (10%)
Finland	34	34 (100%)		0·94	94·3 * (3·5)	24 (71%)	24 (71%)
France	18	18 (100%)		3·5	64·8 (10·3)	16 (89%)	16 (89%)
Germany	58	0		2·41	67·8 (6·7)	0	0
The Netherlands	133	133 (100%)		1·71	78·6 * (7·4)	132 (99%)	132 (99%)
Portugal	13	0		0·63	68·8 (8·2)	11 (85%)	0
Spain	133	16 (12%)		0·94	73·3 (7·0)	132 (99%)	15 (11%)
UK	404	308 (76%)		2·12	69·7 (10·1)	284 (70%)	245 (61%)
USA	786	705 (90%)		1·93	71·2 (9·9)	539 (69%)	467 (59%)
Total	1687	1308 (78%)		1·83	70·1 (9·5)	1216 (72%)	974 (58%)
Controls							
USA (controls from PSA)	2847	0		0·88	NA	2832 (99%)	0
USA (controls from SC)	1523	0		0·78	38 (5·7)	959 (63%)	..
Total	4370	0		0·83	NA	3791 (87%)	0

Data are n (%) or mean (SD), unless stated otherwise. NA=not applicable. PSA=Genetic Analysis of Psoriasis and Psoriatic Arthritis database. SC=Genetic Architecture of Smoking and Smoking Cessation database.

* Represents age at death, which was available for these cohorts; these values were not used for calculation of the complete mean age at onset.

Table 2

Top signals of association at each locus that passed genome-wide threshold for significance and their replication and meta-analysis p values

	General characteristics					Discovery					Replication					Meta-analysis	
	Chromosome	Position	Variant	R^2	Eur_AF*	MA	MAF_A	MAF_U	OR (95% CI)	p value	Power†	MAF_A	MAF_U	OR (95% CI)	p value	OR (95% CI)	p value
Global cohort																	
APOE	19	45 411 941	rs429358	0·949	0·149	C	0·283	0·140	2·40 (2·14–2·7)	$1·05 \times 10^{-88}$	1	0·282	0·148	2·74 (2·15–3·49)	$4·00 \times 10^{-16}$	2·46 (2·22–2·74)	$3·31 \times 10^{-64}$
SNCA	4	90 756 550	rs7681440‡	0·996	0·52	C	0·411	0·483	0·73 (0·66–0·81)	$6·39 \times 10^{-10}$	0·95	0·38	0·47	0·68 (0·56–0·82)	$6·00 \times 10^{-5}$	0·73 (0·67–0·79)	$9·22 \times 10^{-13}$
GBA	1	155 135 036	rs35749011	0·957	0·014	G	0·033	0·014	2·55 (1·88–3·46)	$1·78 \times 10^{-9}$	0·83	0·044	0·022	1·81 (1·05–3·11)	0·033	2·27 (1·75–2·95)	$6·57 \times 10^{-10}$
BCL7C/STX1B	16	30 886 643	rs897984‡	0·984	0·609	T	0·334	0·405	0·74 (0·67–0·82)	$3·30 \times 10^{-9}$	0·96	0·368	0·388	0·98 (0·81–1·19)	0·83	0·77 (0·71–0·85)	$1·19 \times 10^{-8}$
GABRB3	15	26 840 998	rs1426210	0·982	0·315	G	0·348	0·293	1·34 (1·21–1·48)	$2·62 \times 10^{-8}$	0·9	0·281	0·307	0·84 (0·68–1·04)	0·1	1·22 (1·11–1·33)	$2·05 \times 10^{-5}$
Neuropathologically diagnosed cases																	
APOE	19	45 411 941	rs429358	0·949	0·149	C	0·292	0·140	2·52 (2·23–2·85)	$2·77 \times 10^{-49}$	..	..	..	..	..	..	..
SNCA	4	90 756 550	rs7681440‡	0·996	0·52	C	0·409	0·483	0·73 (0·66–0·81)	$2·82 \times 10^{-9}$	..	..	..	..	..	..	..
GBA	1	155 135 036	rs35749011	0·957	0·014	G	0·037	0·014	2·87 (2·10–3·90)	$2·67 \times 10^{-11}$	..	..	..	..	..	..	..
BCL7C/STX1B	16	30 886 643	rs897984‡	0·984	0·609	T	0·332	0·405	0·73 (0·65–0·81)	$4·32 \times 10^{-9}$	..	..	..	..	..	..	..
GABRB3	15	26 840 998	rs1426210	0·982	0·315	G	0·350	0·293	1·34 (1·20–1·45)	$1·21 \times 10^{-7}$	..	..	..	..	..	..	..

R^2=imputation R^2 of each specific variant from Haplotype Reference Consortium. OR=odds ratio. MA=minor allele. MAF_A=minor allele frequency in cases. MAF_U=minor allele frequency in controls.

*Eur_AF is the alternative allele frequency derived from the European population of the Genome Aggregation Database (gnomAD).[23]

†Power refers to the calculated statistical power to replicate the discovery signal, taking into account the replication sample size, effect, and frequency in discovery and an association threshold of p<0·05.

‡Represents variants for which the gnomAD allele frequency corresponds to the alternative allele and not the effect allele.

Table 3

Characteristics of the replication cohort

	Neuropathological diagnosis	Men:women ratio	Age at onset (years)*
USA: patients with dementia with Lewy bodies	350/527 (66%)	2·01	76·3 (8·2)
USA: controls	0/663	0·75	67·8 (10·0)

Data are n (%) or mean (SD), unless stated otherwise.

*Denotes age at examination for controls; for patients with dementia with Lewy bodies, the age reflects age at onset for those diagnosed clinically and age at death for those pathologically diagnosed.

Annex 3

Article: A comprehensive screening of copy number variability in dementia with Lewy bodies.

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A comprehensive screening of copy number variability in dementia with Lewy bodies

A full list of authors and affiliations appears at the end of the article.

Abstract

The role of genetic variability in dementia with Lewy bodies (DLB) is now indisputable; however, data regarding copy number variation (CNV) in this disease has been lacking. Here, we used whole-genome genotyping of 1454 DLB cases and 1525 controls to assess copy number variability. We used 2 algorithms to confidently detect CNVs, performed a case-control association analysis, screened for candidate CNVs previously associated with DLB-related diseases, and performed a candidate gene approach to fully explore the data. We identified 5 CNV regions with a significant genome-wide association to DLB; 2 of these were only present in cases and absent from publicly available databases: one of the regions overlapped *LAPTM4B*, a known lysosomal protein, whereas the other overlapped the *NME1* locus and *SPAG9*. We also identified DLB cases presenting rare CNVs in genes previously associated with DLB or related neurodegenerative diseases, such as *SNCA*, *APP*, and *MAPT*. To our knowledge, this is the first study reporting genome-wide CNVs in a large DLB cohort. These results provide preliminary evidence for the contribution of CNVs in DLB risk.

Keywords

Dementia with Lewy bodies; copy number variants; *MAPT*; *SNCA*; Genome-wide

1. Introduction

Dementia with Lewy bodies (DLB) is a common and complex form of neurodegenerative disease, and its diagnosis can often be complicated by phenotypic similarities with Alzheimer's disease (AD), Parkinson's disease (PD), or even frontotemporal dementia (FTD) (Claassen et al., 2008; Heidebrink, 2002). A more accurate DLB diagnosis is usually obtained by integrating clinical and pathological data from brain autopsy (McKeith et al., 2017).

Genetic studies in DLB have been limited, certainly in comparison with studies on AD or PD, for a number of reasons, most notably because DLB has not been historically

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5.Disclosure

The authors have no actual or potential conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neurobiolaging.2018.10.019.

considered a genetic disease, given the lack of multiplex kindreds where the disease segregates. In addition, large cohorts of patients are difficult to collect given the frequency of the disease and the rate of misdiagnosis. Despite this, recent studies have conclusively shown that there is a role for genetics in the etiology of DLB (Bras et al., 2014; Guerreiro et al., 2018, 2016; Nalls et al., 2013; Peuralinna et al., 2015). Exome sequencing studies have been performed in small cohorts, as have case studies and Sanger sequencing of specific target genes (Clark et al., 2009; Geiger et al., 2016; Keogh et al., 2016; Koide et al., 2002; Ohtake et al., 2004). Copy number variation (CNV) has not been assessed thus far in DLB, particularly in an unbiased manner and at a genome-wide level.

CNVs have been widely studied in a number of neurological conditions, particularly in developmental phenotypes such as schizophrenia and autism (Glessner et al., 2009; Marshall et al., 2017; McCarthy et al,, 2009) where several microdeletions and microduplications (100–600 kb) have been found to be associated with both diseases (Bassett et al., 2017; Cook et al., 1997; McCarthy et al., 2009; Stefansson et al., 2008; Weiss et al., 2008). In these phenotypes, CNVs play a prominent role in the disease genetic architecture.

Several studies have analyzed CNVs in AD, where APP duplications have been unequivocally shown to cause disease (Delabar et al., 1987; Ghani et al., 2012; Swaminathan et al., 2011, 2012; Zheng et al., 2014, 2015). In PD, pathogenic CNVs are also known to occur in *SNCA, PARK2, PINK1*, and *PARK7* (Bonifati et al,, 2003; Chartier-Harlin et al., 2004; Ibanez et al., 2004; Lesage et al., 2008; Marongiu et al., 2007; Waters and Miller, 1994). Together, these data show that CNVs are an important mutational event in neurological conditions.

Here, we report the first genome-wide analysis of CNVs in DLB in a large cohort of patients, many of which with neuropathology diagnoses of DLB. We performed a case-control association study that was complemented by discovery stage analyses guided by candidate genes and CNVs previously reported as being associated with DLB-related neurodegenerative diseases.

2. Materials and methods

2.1. Sample selection

A total of 1454 patients diagnosed with DLB and of European ancestry were selected for this study. Diagnosis of DLB was made according to clinical or pathological criteria (McKeith et al., 2005), Briefly, these included 298 clinically diagnosed and 1156 neuro-pathologically diagnosed cases. Detailed sample and processing information has been described previously (Guerreiro et al., 2018). Data from 1525 control samples were obtained from The Genetic Architecture of Smoking and Smoking Cessation study (phs000404.vl.pl) publicly available at the database of genotypes and phenotypes (https://www.ncbi.nlm.nih.gov/projects/gap/cgi-bin/study.cgi?study_id=phs000404.vl.pl). Supplementary Fig. 1 shows an overview of the study design, different quality control (QC) steps, and analyses performed.

2.2. Genotyping, quality control, and CNV calling

Seven hundred fifty-four DLB samples were genotyped on HumanOmni2.5Exome-8 v1.O.B Illumina arrays, and 700 DLB samples were genotyped using Infinium OmniExpress-24 v1. 2.A1 Illumina arrays (Illumina, Inc, CA, USA). Control samples were genotyped on HumanOmni2.5–4 v1.D arrays (Illumina, Inc, CA, USA). Intensity files were analyzed using GenomeStudio v2011.1 software (Illumina, Inc, CA, USA) along with the respective manufacturer's cluster files. QC procedures were performed in GenomeStudio (GS) before CNV analysis as described by Jarick et al. (Jarick et al., 2014). In short, samples with call rates lower than 0.97 were filtered out. SNP statistics were recalculated following visual inspection of B allele frequency (BAF) and log R ratio (LRR) plots. SNPs with GenTrain scores below 0.7 were excluded. Finally, samples with substantial cryptic reiatedness scores (PLHAT > 0.1) were removed, as previously described (Guerreiro et al., 2018).

CNV calls were generated using 2 different algorithms: cnvPartition v2.3.0 (Illumina, Inc) and PennCNV v1.0.4 (Wang et al., 2007). CNV calling based on cnvPartition was performed by GS with default parameters. For PennCNV, probe positions, LRR, and BAF values for samples that passed QC procedures were exported from GS. Population frequency of the B allele (PFB) files was calculated for each array separately. All smoking cessation samples were used to generate CNVs in cnvPartition and PennCNV, but only a subset of the best performing 700 samples was used for the compilation of the PFB file in PennCNV to match the number of samples used for cases. PennCNV GC-model files were then created based on these PFBs. Finally, CNVs were inferred by PennCNV using the hidden Markov model and the GC-model for wave adjustment. Calls for the X chromosome were generated separately. Chromosome Y SNPs were not analyzed.

2.3. CNV quality control and analysis

To improve the quality of CNVs, only calls generated by both algorithms were kept, whereas calls made by a single algorithm or calls of opposing type (e.g., assigned as a deletion by one algorithm and as duplication by the other) were discarded. Adjacent CNVs were merged if the length of the sequence between them was smaller than 50% of the length of the larger CNV (Mok et al., 2016). CNVs were excluded if they were overlapping telomeres, centromeres, known segmental duplications, the immunoglobulin, or T cell receptor loci. Samples having LRR SD > 0.28, BAF drift > 0.002, waviness factor > 0.04, or having more CNV calls than 3*SD + median were excluded (Marshall et al., 2017; Need et al., 2009).

To identify potentially pathogenic CNVs, we analyzed CNVs spanning known genes. We used the database of genomic variants (DGV) (http://dgv.tcag.ca/dgv/app/home, accessed November 2017) to determine the population frequency of CNVs (MacDonald et al., 2014). This information was complemented with the frequency from clinical samples available in DECIPHER v9.18 (https://decipher.sanger.ac.uk/, accessed November 2017).

2.4. Case-control association analysis

Case-control association analysis was implemented using ParseCNV (Glessner et al., 2013). Standard ParseCNV quality metrics were used to filter out low-quality results. CNVs that were genome-wide significant [p-value $< 5 \times 10^{-4}$ as suggested by (Glessner etal., 2013)]

had a minimum length of 50 kb, and passed visual inspection in GS were selected for further analyses.

2.5. Candidate CNVs approach

CNVs previously described in AD (Ghani et al., 2012; Heinzen et al., 2010; Swaminathan et al., 2011, 2012; Zheng et al., 2014, 2015), PD (Bademci et al., 2010; Liu et al., 2013; Mok et al., 2016; Pankratz et al., 2011), and FTD (Gijselinck et al., 2008) were specifically investigated in these data (Supplementary Table 1). This analysis was performed on the complete set of CNV results after QC, disregarding the filters used for the case-control association analysis performed by ParseCNV.

2.6. Candidate genes approach

CNVs located in known AD, PD, FTD, and DLB genes were also assessed (Brás etal., 2015; Guerreiro et al., 2013,2015, 2018; Jansen et al., 2015; Keogh et al., 2016; Koide et al., 2002; Ohtake et al., 2004; Saitoh et al., 1995). Supplementary Table 2 lists all genes studied using this approach.

3. Results

3.1. CNV calling and QC steps

After QC steps at the GS level, a total of 2819 samples (1294 cases and 1525 controls) remained for further analyses. From the 754 DLB samples genotyped with HumanOmni2.5 arrays, 616 (81.7%) samples were kept and from the 2,567,845 probes in this array, 2,496,600 (97.2%) passed QC. Six hundred seventy-eight (96.9%) samples of the 700 samples genotyped with OmniExpress arrays passed QC and from the 713,599 probes available in this chip, 698,680 (97.9%) probes remained. All controls from the Smoking Cessation database had good quality genotypes (call rate > 0.97) and, consequently, no samples were excluded, and 2,390,384 (97.8%) of the 2,443,177 probes were kept.

After combining the results obtained by the 2 CNV calling algorithms (cnvPartition and PennCNV), excluding samples because of their relatedness, and performing the PennCNV QC steps on the LRR and BAF values and number of calls, a final number of 2615 individuals (1187 DLB cases and 1428 control samples) and 80,416 CNVs were analyzed. All samples carrying CNVs were confirmed to be of European/North American ancestry by merging their data with that of 1000 Genomes.

3.2. Case-control association analysis

Of the 494 CNV regions resulting from the ParseCNV analysis, only 5 passed QC checks and were statistically significant (Table 1). Of these, 2 of the regions were not present in our control population or in public databases: the deletion overlapping *LAPTM4B* ($p = 6.29 \times 10^{-7}$) and the one overlapping *SPAG9-NME1-NME2* ($p = 2.72 \times 10^{-4}$) (Fig. 1).

3.3. Candidate CNVs approach

Five CNVs previously associated with DLB-related neurodegenerative diseases were found in DLB patients (Table 1). Two of these were present in the control group with a higher

frequency than in the patient group, and the remaining 3 are described in public databases and are detailed next. The duplication identified on chromosome 12 overlapping *DDXU* and *OVOS2* has a frequency 10-fold higher in DGV than in the DLB cohort. The 16pl 1.2 microduplication found in one DLB patient has a frequency of 1.69×10^{-4} in the DECIPHER database but does not occur in any control samples or in the DGV database. One of the CNVs that was previously significantly associated with AD locates at chr8:2,792,874–4,852,328 and overlaps *CSMD1* (Swaminathan et al., 2011). *CSMD1* has frequent deletions and duplications spanning the whole gene as reported in DGV. Our results agree with this: at this locus, we identified over 100 deletions in cases and controls. One region in the gene has a deletion in 3 cases and in no controls and this CNV showed a suggestive significance in our DLB case-control association analysis. Therefore, we only report this shorter region in Table 1.

3.4. Candidate genes approach

We investigated CNVs in genes known to be associated with diseases that are related to DLB (Supplementary Table 2) and identified a total of 8 CNVs (Table 1). These included one duplication in *APP* occurring in a clinically diagnosed case. This large duplication is not present in the databases or in the control cohort. Two DLB subjects were found to carry duplications spanning *MAPT*, and one neuropathologically diagnosed patient was found to cany a *SNCA* duplication (Fig. 2). *PARK2* was found to have many copy number losses and gains in controls (n = 28) and cases (n = 13) but none were homozygous. A duplication including *CHCHD10* was also identified in a neuropathologically diagnosed DLB patient. No CNVs were identified in *CBA*, the gene presenting the strongest association with DLB.

4. Discussion

We performed a systematic analysis of CNVs in a large cohort of DLB patients using 3 main approaches. The first of these approaches was a case-control association analysis, which resulted in 5 significant CNV regions that have not been previously described as associated with the disease. The most significant result from this analysis was a deletion spanning a lysosome-associated transmembrane protein, *LAPTM4B*. Intraneuronal alpha-synuclein clearance likely occurs through a variety of mechanisms to maintain protein homeostasis. However, recent data have highlighted the importance of lysosomal pathways for degradation of this protein (Webb et al., 2003). Interestingly, a member of the same protein family, *LAPTM5*, was one of the top hits for incidental DLB in a recent network analysis study (Santpere et al., 2018). Although we cannot directly link this CNV to the development of DLB in these cases, it is interesting that a lysosomal enzyme is the top hit in our association analysis, given the prominent role of the lysosome in Lewy body diseases. In fact, a strong GWAS hit for DLB is *GBA*, a gene involved in lysosomal lipid storage disorders.

Also associated with DLB and absent from publicly available databases was a deletion overlapping the*NME1* locus.*NME1* is involved in purine metabolism, which has been reported to be disrupted in AD, PD, and Creutzfeldt—Jakob disease. *NME1* mRNA was also found to be reduced in these diseases (Ansoleaga etal.,2015,2016; Garcia-Esparcia et al.,

2015). Here, we identified a deletion at the 3'-end of *NME1*, which could be consistent with a reduced expression of the gene in DLB, although this was not tested in the present study.

Using a candidate gene approach where we analyzed genes known to have a role in DLB and DLB-related diseases, we identified several CNVs of potential interest. The hallmark of DLB at autopsy is the accumulation of alpha-synuclein protein within neurons and their processes, termed Lewy bodies and Lewy neurites (Spillantini et al., 1997). Variants in the *SNCA* gene, which encodes alpha-synuclein, have been previously associated with the risk of developing DLB (Bras et al., 2014; Guerreiro et al., 2018). In addition to point mutations, CNVs including *SNCA* are known to cause PD, and over the past years, evidence has suggested that this gene may also be duplicated in DLB. Nishioka et al. identified a PD family with a duplication spanning all of *SNCA* and *MMRN1* where the proband was later neuropathologically diagnosed as DLB (Nishioka et al., 2006; Obi et al., 2008). Four neuropathologically diagnosed DLB cases presented a large duplication from *DSPP* to *PDLIM5* including *SNCA*, 3 of these were heterozygous and 1 was homozygous (Ikeuchi et al., 2008). A duplication in *SNCA* was also described in a probable DLB patient in a study with 99 cases (Meeus et al., 2012). Here, we add to this body of evidence, by identifying another patient neuropathologically diagnosed with DLB carrying a SNCA duplication. In our DLB cohort, this duplication shows a similar frequency to that provided by DGV. However, the frequency reported in DGV results from 2 entries in that database. When looking in more detail at these 2 entries, they are actually duplicated entries from the same Human Genome Diversity Project (HGDP) sample from Cambodia (HGDP00721). Given that information for each HGDP samples is limited to sex of the individual, population, and geographic origin, it is possible this sample originated from a PD or DLB patient or from an asymptomatic carrier, as these have previously been reported, with SNCA multiplications having particularly low penetrance levels in Asian populations (Ahn et al., 2008; Nishioka et al., 2006). It is also possible the duplication reported is an artifact caused by the creation or passage of the lymphoblast cell lines used to extract DNA (Simon-Sanchez et al., 2007).

We also identified one heterozygous duplication encompassing *CHCHD10*, a gene previously shown to cause FTD and/or amyotrophic lateral sclerosis (Zhang et al., 2015). However, given that disease-associated *CHCHD10* mutations are loss of function (Perrone et al., 2017), it is not clear whether a duplication of the gene would be pathogenic.

GABRB3 is a gamma-aminobutyric acid (GABA) receptor that was reported to be associated with DLB in a recent GWAS but did not survive independent replication (Guerreiro et al., 2018). However, loss of GABA receptors could underlie the typical visual hallucinations in DLB (Khundakar et al., 2016), and because of this, we specifically looked at CNVs in *GABRB3* and identified a duplication in one case. Given the GWAS results previously mentioned, the CNV detected here and the fact that GABA receptor neurotransmission is altered in DLB (Santpere et al., 2018), it is tempting to speculate that genetic variability in GABA receptors may, in fact, modulate risk for DLB.

We identified 2 clinically diagnosed DLB samples with *MAPT* duplications (Fig. 3). *MAPT* was not found to be significantly associated with DLB in recent GWAS (Bras et al., 2014; Guerreiro et al., 2018), but the *MAPT* HI haptotype was previously described as a possible

risk factor for DLB (Cervera-Carles et al., 2016; Labbé et al., 2016) and is a well-known genetic risk factor for PD. Previous studies of small cohorts of FTD patients have not revealed causative *MAPT* duplications (Lladó et al., 2007; Skoglund et al,, 2009) but the screening of French FTD patients including multiplex families led to the identification of a heterozygous partial deletion of *MAPT* (Rovelet-Lecrux et al., 2009) and a 17q21.31 microduplication in an atypical FTD case (Rovelet-Lecrux et al., 2010). More recently, *MAPT* duplications were shown to increase expression of *MAPT* mRNA and were found to cause tangle pathology without Aβ deposition in probable AD patients (Le Guennec et al., 2017).

PARK2 homozygous CNVs are the most common copy number cause of PD, accounting for more than 50% of all pathogenic mutations in the gene and more frequently affecting the region between exons 2 and 7 (Hedrich et al., 2004; Kim et al., 2012). Our results showed no significant differences in the frequency of heterozygous CNVs overlapping *PARK2* between DLB cases and controls, similar to the findings by Kay et al. in PD (Kay et al., 2010). In addition, we did not find any homozygous *PARK2* CNVs suggesting that CNVs in this gene do not play a causative role in DLB.

APP duplications are known to cause AD (Delabar et al., 1987; Ghani et al., 2012; Swaminathan et al., 2011, 2012; Zheng et al., 2014, 2015). The sample carrying an *APP* duplication in our cohort has a clinical diagnosis of DLB without neuropathological confirmation; it is therefore possible that this is an AD case misdiagnosed as DLB. However, there have been reports in the literature of DLB cases associated with *APP* duplications. For example, in a French family presenting with a diverse phenotype, *APP* duplication was associated with DLB confirmed by neuropathological findings (Guyant-Marechal et al, 2008). Similarly, one case with Lewy body—variant AD was reported in a multigenerational dementia family from the Netherlands (Sleegers et al., 2006). DLB cases frequently present Aβ pathology at autopsy (Hepp et al., 2016), and it has been suggested that Aβ accumulation can trigger Lewy body disease (Masliah et al, 2001).

There are 2 main limitations in this study: first, this is a relatively small-sized cohort, which means we cannot confidently assess associations of CNVs with low effect sizes on disease; second, we did not perform independent replication of these findings, which precludes us from establishing definite associations or causes of disease. Despite these limitations, we report on the first systematic analysis of CNVs in a large cohort of DLB patients, using well-established analytical practices. We identified potential disease causing CNVs as well as potential novel candidate genes for DLB. Despite this, our results suggest that it is unlikely that CNVs play a significant role in the pathogenesis of DLB.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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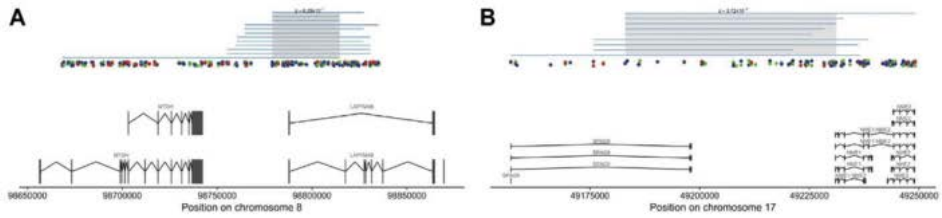

Fig. 1.

Schematic representation of statistically significant CNVs resulting from the case–control analysis that were not found in controls or publicy available databases. CVNs overlappig *LAPTM4B* in A; and *SPAG9/NME1* in B.Known transcripts are represented at the bottom of each panel. Passing QC SNPs used for the CNV calling are depicted by color according to the genotyping arrary (red: OmniExpress, blue: Omni2.5 M, green: Omni2.5 M for controls). CNVs (duplications) are depicted as bars above the genes. The gray shadow area represents the associated region that is genome-wide significant.

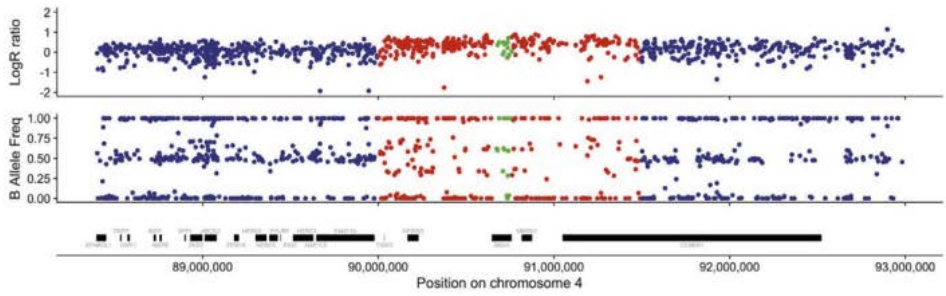

Fig. 2.

Duplication identified at the *SNCA* locus. Log R ratio and B alleie frequency plots of the CNV identified in the *SNCA* locus. Each point represents an SNP according to location in chromosome 4 (position on X axis). The genomic duplication is indicated by an increase in log R ratio and B allele frequency clusters outside the expected values of 1 (B/B), 0.5 (A/B), and 0 (A/A). Genes are represented at the bottom as black bars. SNPs inside the CNV region are represented in red. SNPs outside the CNV region are represented in blue, and SNPs in SNCA are represented in green.

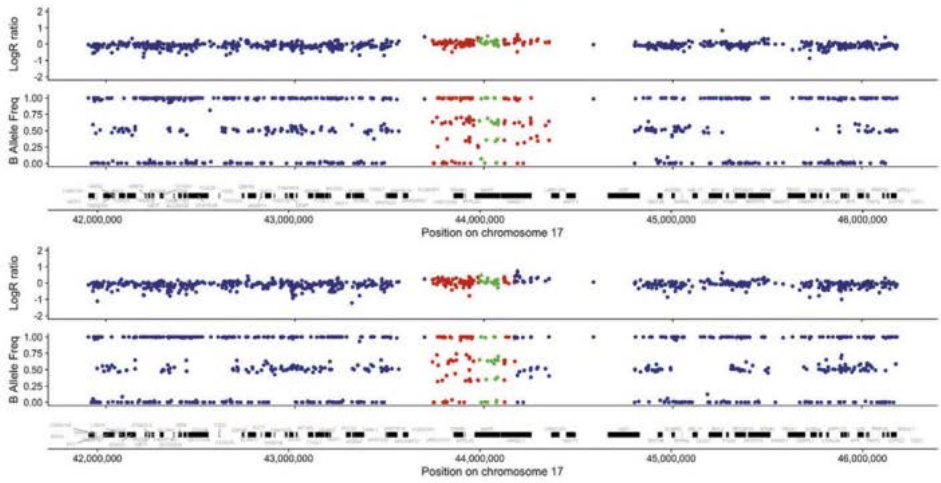

Fig. 3.

Duplications identified at the *MAPT* locus. Log R ratio and B allele frequency plots of the CNVs identified at the *MAPT* locus in 2 clinically diagnosed DLB cases. Each point represents an SNP according to location in chromosome 17 (position on X axis). The genomic duplication is indicated by an increase in log R ratio and B allele frequency clusters outside the expected values of 1 (B/B), 0.5 (A/B), and 0 (A/A). Genes are represented at the bottom as black bars. SNPs inside the CNV regions are represented in red, SNPs outside the CNV regions are represented in blue, and SNPs in *MAPT* are represented in green.

Table 1

Results from the three types of analyses

Genes	Location	CNV	*p*-value	Cases (Neuro[a])	Controls	Cases frequency	Controls frequency	DGV frequency	DECIPHER frequency
Case-control association analysis									
ADGRG7,TFG	chr3:100,357,671–100,439,759	Gain	8.93×10^{-5}	21 (18)	13	1.77×10^{-2}	9.10×10^{-3}	1.43×10^{-2}	2.09×10^{-2}
PDZD2	chr5:32101,400–32,106,628	Gain	2.94×10^{-6}	14 (12)	0	1.18×10^{-2}	0	3.00×10^{-4}	1.69×10^{-4}
LAPTM4B	chr8:98755,434–98,800,334	Loss	6.29×10^{-7}	12 (6)	0	1.01×10^{-2}	0	0	0
MSR1	chr8:15948,235–16,021,468	Loss	1.20×10^{-4}	13 (7)	4	1.10×10^{-2}	2.80×10^{-3}	4.30×10^{-3}	7.10×10^{-3}
NME1,NME1-NME2,SPAG9	chr17:49,177,096–49,231,786	Loss	2.72×10^{-4}	9 (4)	0	7.58×10^{-3}	0	0	0
Candidate CNVs approach									
CSMD1	chr8:4,033,908–4,126,540	Loss	2.89×10^{-2}[b]	3 (2)	0	2.53×10^{-3}	0	3.00×10^{-4}	0
DDX11,OVOS2	chr12:31,249,834–31,407,303	Gain	1.41×10^{-2}[b]	4 (2)	0	3.37×10^{-3}	0	4.61×10^{-2}	0
CYFIP1,GOLGA8I,NIPA1,	chr15:22,750,305–23,272,733	Gain	na	5 (5)	8	4.21×10^{-3}	5.60×10^{-3}	2.50×10^{-3}	1.86×10^{-3}
NIPA2,TUBGCP5,WHAMML1									
CHRNA7,OTUD7A	chr15:31,932,865–32,515,849	Loss or Gain	na	6 (5)	13	5.05×10^{-3}	9.10×10^{-3}	6.90×10^{-3}	8.79×10^{-3}
ASPHD1,BOLA2,C16orf54,CDIPT,CDIPT-AS1,	chr16:29,595,483–29,912,902	Gain	na	1 (1)	0	8.42×10^{-4}	0	0	1.69×10^{-4}
KIF22,MAZ,MVP,PAGR1,PRRT2,QPRT,SEZ6L2,									
SPN,ZG16									
Candidate genes approach									
DNAJC6,LEPR,LEPROT	chr1:65,854,556–65,955,725	Gain	na	1 (0)	1	8.42×10^{-4}	7.00×10^{-4}	1.72×10^{-4}	2.53×10^{-3}
SNCA,SNCA-AS1,GPRIN3,MMRN1,CCSER1	chr4:90,035,549–91,420,358	Gain	na	1 (1)	0	8.42×10^{-4}	0	6.42×10^{-4}	0
PARK2	chr6:161,601,162–163,259,260	Loss or Gain	na	13 (8)	28	1.10×10^{-2}	1.96×10^{-2}	2.30×10^{-3}	1.69×10^{-1}
GABRB3,GABRA5,GABRG3	chr15:26,996,126–27,220,713	Gain	na	1 (1)	0	8.42×10^{-4}	0	0	1.18×10^{-3}
MAPT,CRHR1,KANSL1,KANSL1-AS1,	chr17:43,661,362–44,345,063	Gain	na	2 (0)	0	1.68×10^{-3}	0	1.03×10^{-4}	1.18×10^{-3}
MAPT-AS1,MAPT-IT1,SPPL2C,STH									
APPADAMTS1,ADAMTS5,ATP5J,CYYR1,	chr21:25,063,840–28,522,487	Gain	na	1 (0)	0	8.42×10^{-4}	0	0	0
CYYR1-AS1,GABPA,JAM2,MRPL39									
CHCHD10,ADORA2A,ADORA2A-AS1,	chr22:23,690,325–25,011,417	Gain	na	1 (1)	0	8.42×10^{-4}	0	6.00×10^{-4}	1.18×10^{-3}
C22orf5,CABIN1,CES5AP1,DDT,DDTL,									

Genes	Location	CNV	*p*-value	Cases (Neuro[a])	Controls	Cases frequency	Controls frequency	DGV frequency	DECIPHER frequency
DERL3,DRICH1,GGT1,GGT5,GSTT1,GSTT1-AS1, *GSTT2,GSTT2B,GSTTP1,GSTTP2,GUCD1,* *GUSBP11,IGLL1,LRRC75B,MIF,MIF-AS1,* *MMP11,POM121L9P,RGL4,SLC2A11,SMARCB1,* *SNRPD3,SPECC1L,,SUSD2,UPB1,VPREB3,* *ZDHHC8P1,ZNF70*	chr22:42,522,613–42,531,210	Loss or Gain	na	1 (1)	4	8.42×10^{-4}	2.80×10^{-3}	2.20×10^{-1}	3.00×10^{-2}
CYP2D6,NDUFA6-AS1									

All genomic coordinates are for the genome assembly hg19.

[a] Neuropathological diagnosis; na–not applicable; *p*-value—these were calculated including all cases (not only neuropathologically confirmed cases),

[b] *p*-values resulting from the case-control association analysis.

Annex 4

Article: GBA and APOE ε4 associate with sporadic dementia with Lewy bodies in European genome wide association study.

SCIENTIFIC REPORTS

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GBA and *APOE* ε4 associate with sporadic dementia with Lewy bodies in European genome wide association study

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Dementia with Lewy Bodies (DLB) is a common neurodegenerative disorder with poor prognosis and mainly unknown pathophysiology. Heritability estimates exceed 30% but few genetic risk variants have been identified. Here we investigated common genetic variants associated with DLB in a large European multisite sample. We performed a genome wide association study in Norwegian and European cohorts of 720 DLB cases and 6490 controls and included 19 top-associated single-nucleotide polymorphisms in an additional cohort of 108 DLB cases and 75545 controls from Iceland. Overall the study included 828 DLB cases and 82035 controls. Variants in the *ASH1L/GBA* (Chr1q22) and *APOE ε4* (Chr19) loci were associated with DLB surpassing the genome-wide significance threshold ($p < 5 \times 10^{-8}$). One additional genetic locus previously linked to psychosis in Alzheimer's disease, *ZFPM1* (Chr16q24.2), showed suggestive association with DLB at p-value $< 1 \times 10^{-6}$. We report two susceptibility loci for DLB at genome-wide significance, providing insight into etiological factors. These findings highlight the complex relationship between the genetic architecture of DLB and other neurodegenerative disorders.

Dementia with Lewy Bodies (DLB) is the second most common type of neurodegenerative dementia, accounting for 15% of dementia patients. DLB overlaps clinically, pathologically and genetically with Alzheimer's disease (AD) and Parkinson's disease (PD). Clinically, DLB is characterized by cognitive impairment, parkinsonism, psychotic symptoms like visual hallucinations, fluctuating cognition with pronounced variations in attention and alertness and REM sleep behaviour disorder. Reduced uptake on CIT-SPECT or myocardial scintigraphy and polysomnography with confirmation of REM sleep without atonia have been included as indicative biomarkers in the diagnostic criteria[1]. We have previously shown the clinical diagnostic criteria for probable DLB to be both sensitive (77%) and highly specific (94%) as compared to a pathological DLB diagnosis. Furthermore, we have found DLB to have higher costs, more neuropsychiatric symptoms, a more rapid cognitive decline, shorter time until nursing home admission, shorter survival and higher caregiver distress as compared to AD[2–8].

In some families, DLB occurs with autosomal dominant heritance at an age of onset <65 years. In these families, alpha-synuclein (*SNCA*) multiplications or point mutations have been described[9,10]. DLB is however typically late onset (i.e. onset after 65 years of age) and sporadic, and the proportion of phenotypic variance that can be explained by >250,000 SNPs on the NeuroX array has been estimated to 31% with substantial genetic overlap with both AD and PD[11]. Indeed, previous genetic studies have suggested associations of *APOE*, *GBA*, *SNCA* and *SCARB2* with DLB in both neuropathologically and clinically diagnosed cases[12]. Data from another GWAS of DLB were recently presented and confirmed *APOE e4*, *SNCA* and *GBA*, and in addition suggested *CNTN1* to be associated with DLB[13]. Regarding *APOE*, the strongest genetic risk factor for AD, we showed that the *APOE ε4* allele increases and the *APOE ε2* allele decreases the risk of developing DLB[14]. *GBA*, the gene encoding the lysosomal enzyme glucocerebrosidase, is associated with PD risk and cognitive decline in PD[15,16]. In DLB, *GBA* mutations have been reported in 7.8% of cases (odds ratio (OR) ~8), even up to 31% in Ashkenazi Jews, suggesting that *GBA* is a stronger risk factor for DLB than for PD[17]. Moreover, whole exome sequencing studies have identified rare and pathogenic variants in *GBA*, *PSEN1* or *APP* in 4.4–25% of patients with pathologically or clinically diagnosed DLB[18,19]. However, none of the AD associated common variants identified in large genome-wide association studies (GWAS) have been associated to DLB besides *APOE*.

Notwithstanding these interesting results from early genetic studies, the individual genetic risk factors that specifically contribute to the common and sporadic late onset form of DLB have remained relatively unexplored compared to PD and AD, largely due to the lack of large sample series providing adequate statistical power for GWAS. In the current study, we collected DNA and genotyped samples from the Norwegian DemGene consortium and the European DLB consortium (E-DLB), and performed a GWAS applying a two-stage meta-analysis approach and follow-up in an independent cohort. We investigated whether common genetic variants are associated with DLB, aiming to elucidate the molecular mechanisms underlying the disease.

Methods

Participants. Three discovery cohorts were included in the study (Cohorts 1, 2 and 3). We collected samples from the Norwegian Dementia Genetics Network (DemGene) and from the European DLB consortium (E-DLB). Cohort 1 included DemGene and three European centres (Strasbourg, Amsterdam and Lund), and consisted of 478 cases and 1322 controls. An additional Norwegian population cohort of 4875 controls was added. Cohort 2 included two European centres both from Barcelona and consisted of 242 cases and 293 controls. Cohort 3 samples were collected in Iceland and consisted of 108 cases and 75545 controls. Altogether 828 DLB cases and 82110 controls were included in this study, see Supplementary material and Supplemental (S.) Table 1 for details. All research was performed in accordance with relevant guidelines/regulations, and informed consent was obtained from all participants and/or their legal guardians.

Genotyping. DNA was extracted from whole blood. We genotyped Cohort 1 on the Illumina Infinium Omni Express-24 v1.1 platform (Illumina Inc., San Diego, CA, USA) at deCODE Genetics (Reykjavik, Iceland) in concordance with the standard Illumina protocol. We genotyped Cohort 2 samples with the Illumina Infinium Omni Express Exome-8v1.3 chip. Cohort 3 samples were genotyped on Illumina's HumanHAP300, HumanHAP300-Duo and HumanCNV370 bead arrays. We conducted assignment of genotypes according to the standard Illumina protocol in GenomeStudio software V2011.1 version 1.9.4. We tested for plate effects and other batch effects by a number of association tests described in detail under supplementary methods. Markers exhibiting high rates of genotyping missingness (above 5%), minor allele frequency (MAF) below 1% or showing departure from Hardy Weinberg equilibrium ($p < 1 \times 10^{-4}$ calculated for controls) were excluded from the

analyses. Individuals showing high rates of genotyping missingness (above 5%), cryptic relatedness (pairwise Identity-By-Descent PI_HAT above 20%) or genome-wide heterozygosity (outside mean ± 5 SD of the sample) were removed from the analyses. Further, sex-check was performed based on the homozygosity estimate of X chromosome markers implemented in PLINK.

Association analysis. We performed association analysis in two stages. Due to data regulations and ethical approvals regarding data sharing, we performed genome-wide association analyses on Cohorts 1 and 2 independently and combined the results through meta-analysis to obtain Stage 1 results. HRC imputation was not accessible for cohort 1 due to national regulations in Norway. To reduce possible genomic inflation or overcorrection, the results from the Stage 1 meta-analysis were corrected for genomic inflation before we performed meta-analysis with Cohort 3 to obtain Stage 2 results.

Genotypes from Cohort 1 samples were imputed onto the European reference haplotypes from the 1000 Genomes Project (GRCh37/hg19 assembly) Phase 3 using MACH (http://www.sph.umich.edu/csg/abecasis/MACH). We excluded variants with MAF lower than 0.01 or R-squared quality metric (INFO) > 0.5. We performed principal component analysis (PCA) on Cohort 1 pre-imputation data using PLINK 1.9 (https://www.cog-genomics.org/plink2) to account for population stratification. The association analysis by logistic regression on dosage data using PLINK 1.9 included gender, age and the two first principal components as covariates. Genomic inflation factors were calculated as the ratio of the median of the empirically observed distribution of the association chi-square statistic to the expected median[20].

Genotypes from Cohort 2 samples were imputed onto the GRCh37/hg19 assembly with ShapeIT & Minimac3 using the haplotype reference consortium HRC version r1.1 reference data at the imputation server of the University of Michigan. PCA was done independently for Cohort 2 because there was no relatedness to samples from Cohort 1. Logistic regression was performed using PLINK 1.9 using gender, age and the top two genetic principal components as covariates. The genomic inflation factor was calculated as previously described.

To obtain Stage 1 results, variants from Cohort 1 and Cohort 2 were mapped to each other using GRCh37/hg19 assembly. All variants with allele discrepancies across cohorts were discarded. We performed meta-analysis of Cohort 1 and Cohort 2 using PLINK 1.9 with fixed effects inverse-variance weighted effect sizes. Biases from different cohorts due to genotype array and imputation procedures are mitigated through correction on the inflation factor. The results were verified using METAL meta-analysis tool (http://csg.sph.umich.edu/abecasis/Metal). To identify independently associated loci, we used FUMA's SNP2GENE function to define lead SNPs and genomic risk loci[21]. Graphical representations including quantile-quantile plots and Manhattan plots were performed in R using the qqman package (http://cran.r-project.org/web/packages/qqman).

We selected variants with Stage 1 meta-analysis p-value $< 1 \times 10^{-6}$ for follow up in Cohort 3 (Iceland) and used the same approach described above to meta-analyse the results in Stage 2[22].

Functional mapping and annotation (FUMA) of GWAS. We utilized FUMA to functionally annotate our Stage 1 results[21]. FUMA incorporates 18 biological data repositories such as the Genotype-Tissue Expression (GTEx), the Encyclopedia of DNA Elements (ENCODE), the Roadmap Epigenomics Project and chromatin interaction information. FUMA requires GWAS summary statistics and its outputs include multiple tables and figures containing extensive information on, e.g., functionality of SNPs in genomic risk loci, including protein-altering consequences, gene-expression influences, open-chromatin states as well as three-dimensional (3D) chromatin interactions. Functionally annotated variants are subsequently mapped to prioritized genes based on (i) physical position mapping on the genome, (ii) expression quantitative trait loci (eQTL) mapping and (iii) 3D chromatin interactions (chromatin interaction mapping). Biological information for each prioritized gene is provided to gain insight into previously associated diseases. On top of the single gene level analyses, FUMA also provides information on association overrepresentation in sets of differentially expressed genes (DEG) to identify tissue specificity of prioritized genes. We refer to the details of methods and repositories of FUMA in[21].

Ethics committee approval. All cohorts and sites providing samples for this study have local ethics approval for DNA collection and data sharing, and the names of local ethics committees are provided in the in the supplemental materials. In Norway the joint study was approved by the Regional Committees for Medical and Health Research Ethics in Mid Norway.

Results

From Cohort 1, we obtained genotypes for 719,755 SNPs and performed imputation to obtain 7,769,477 high-quality variants. We performed association using 478 DLB cases and 1322 controls, see S. Table 1. After controlling for population stratification using PCA (S. Fig. 1A), the genomic inflation factor Lambda was 1.005 (S. Fig. 2A). We found genome-wide significance on rs2230288 (closest gene *GBA*, $p = 3.77 \times 10^{-8}$) and rs429358 (closest gene *APOE*, $p = 3.21 \times 10^{-9}$). The regional association plots for this locus is visualized in S. Fig. 3.

To increase the power of our study, we included additional Norwegian population controls in the study, see S. Table 1. Using two principal components (S. Fig. 1B), the addition of population controls increased inflation to a Lambda of 1.244, possibly due to inflation from the additional controls. We verified the inflation using LD Score Regression and found the intercept at 1.2094, consistent with Lambda. Quantile-quantile plots for Cohort 1 before and after genomic correction are given in S. Fig. 2. After correction, the strongest associations in Cohort 1 remain with rs2230288 ($p = 1.77 \times 10^{-10}$) and rs429358 ($p = 4.13 \times 10^{-9}$).

We found 45 SNPs associated to DLB at $p < 5 \times 10^{-6}$ with strong associations in Chromosomes 1 and 19; a summary of our findings from Cohort 1 is given in S. Table 2A.

analyses. Individuals showing high rates of genotyping missingness (above 5%), cryptic relatedness (pairwise Identity-By-Descent PI_HAT above 20%) or genome-wide heterozygosity (outside mean ± 5 SD of the sample) were removed from the analyses. Further, sex-check was performed based on the homozygosity estimate of X chromosome markers implemented in PLINK.

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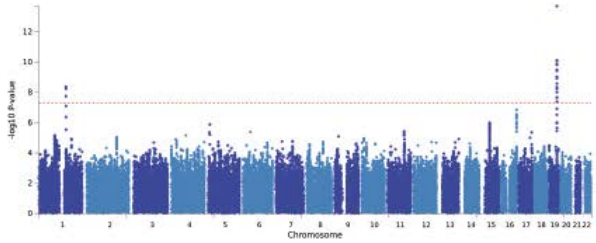

Figure 1. Manhattan plot of Stage 1 meta-analysis. Manhattan plot of meta-analysis of Cohorts 1 and 2 for genome-wide association with Dementia with Lewy Body (DLB). Genome-wide significant associations to DLB (threshold $P < 5 \times 10^{-8}$) are found in chromosomes 1 (*ASH1L/GBA*) and 19 (*APOE*), and a suggestive association to DLB at $P < 1 \times 10^{-6}$ is identified at chromosome 16 (*ZFPM1*). A comprehensive result of Stage 1 is presented in Supplementary Table 2.

From Cohort 2, we analysed 7,570,659 successfully imputed variants. The genomic inflation factor Lambda was 1.031. Quantile-quantile plots for Cohort 2 are given in S. Fig. 2. The Cohort 2 study revealed 9 SNPs associated to DLB at $p < 5 \times 10^{-6}$; also with strong associations in Chromosome 19, see S. Table 2B. After individual analyses of discovery Cohorts 1 and 2, we performed a Stage 1 meta-analysis of 6,963,063 variants (898 were discarded due to allele mismatches). The meta-analysis genomic inflation factor Lambda was 0.865, possibly due to overcorrection for the genomic inflation in Cohort 1. We corrected the chi-square statistics of the meta-analysis at fixed ORs, see the quantile-quantile plots in S. Fig. 2. Genome-wide Stage 1 results are visualized as a Manhattan plot in Fig. 1.

After correction, Stage 1 analysis revealed 108 SNPs associated with DLB at $p < 5 \times 10^{-6}$ (S. Table 2C). The statistical power of our study is estimated to be 0.085 (MAF = 0.05) to 0.395 (MAF = 0.1) to 0.823 (MAF = 0.2) for SNPs with genomic risk ratio GRR = 1.5 and GRR = 1.2, shown in S. Fig. 4. GRR values were chosen based on ORs of discoveries of earlier DLB studies[13]. We followed up on 18 of these SNPs, which were successfully analysed in an independent sample from Iceland (Cohort 3) and performed a Stage 2 meta-analysis. Because stage 2 meta-analysis included only 18 selected SNPs instead of a genome-wide analysis, this result was not corrected for genomic inflation.

From the Stage 2 meta-analysis, we found two susceptibility regions associated with DLB surpassing genome-wide significance, $p < 5 \times 10^{-8}$. We found *APOE ε4* related SNPs at genome-wide significance, represented by rs429358 (OR = 2.28, $p = 6.15 \times 10^{-17}$, see Table 1 for details). A regional association plot of the *APOE* locus from Stage 1 meta-analysis is presented in S. Fig. 5A. From a recent large study, we found that this SNP is identical to the reported top hit (OR = 2.40, $p = 1.05 \times 10^{-48}$) in Guerreiro *et al.*[13].

We also discovered a DLB-associated locus on Chromosome 1, represented by rs12734374 (closest gene: *ASH1L*, OR = 4.31, $p = 1.33 \times 10^{-9}$, see Table 1 and regional association plots in S. Fig. 5B). This SNP is located in the same genomic region of rs2230288, the strongest hit in Cohort 1 which was not successfully imputed in Cohort 2, but had implicated *GBA*. Furthermore, in another study, we found that rs12734374 is in high LD ($R^2 = 0.79$) with a *GBA* hit, rs35749011 (OR = 2.27, $p = 6.57 \times 10^{-10}$ in)[13]. Both our *APOE* and *ASH1L/GBA* hits provide genome-wide significant confirmations of the findings from Guerreiro *et al.*[13].

Furthermore, we investigated SNPs with a suggestive association to DLB. From the Stage 1 meta-analysis, we noted 9 SNPs at $p < 1 \times 10^{-6}$ in chromosome 16, represented by rs12926163 (closest gene *ZFPM1*, OR = 1.68, $p = 1.45 \times 10^{-7}$). These SNPs were not successfully analysed in the Icelandic cohort, and therefore we present only the Stage 1 result of this locus in Table 1 and S. Fig. 5C.

Next, we analysed specific gene signals reported previously in DLB for their significance under locus-wide Bonferroni correction for each gene. Due to the small SNP coverage in the Stage 2 analysis, we used results from Stage 1. We extracted variant information for *SNCA* (GRCh37hg19 chr4: 90,645,250-90,759,466), *SCARB2* (chr4:77,079,886-77,155,689), *MAPT* (chr17:43,971,748-44,105,700) and *CNTN1* (chr12:41,086,244-41,466,220) with upstream and downstream flanking of 200kB. Regional plots of these candidate genes are shown in S. Fig. 5D–G.

Among the 1509 successfully imputed SNPs in the *SNCA* locus, the strongest association was with rs2301135 (chr4:90,758,389, $p = 5.68 \times 10^{-5}$, OR = 1.40, minor allele C) and remained nearly significant after correction (threshold $p < 3.3 \times 10^{-5}$) using conservative multiple test assumptions of independent SNPs. In the *SCARB2* locus, the strongest association among the 1600 SNPs was with rs34216031 (chr4:76,971,832, OR = 1.63, $p = 1.37 \times 10^{-2}$), but its significance did not survive correction (threshold $p < 3.1 \times 10^{-5}$). In the *MAPT* locus, 694 SNPs passed quality checks. Among these, the strongest association was with rs11652003 (chr17:44,132,659, OR = 0.75, $p = 1.89 \times 10^{-2}$) but did not withstand correction (threshold $p < 7.2 \times 10^{-5}$). Of note, coverage of *MAPT* is relatively poor in our genotyping and imputation procedure, see S. Fig. 5F. In the *CNTN1* locus, the strongest association was with rs56260639 (chr12:41122583, OR = 0.50, $p = 1.17 \times 10^{-2}$). Despite the strong OR, this association did not remain significant after correction (threshold $p < 2.1 \times 10^{-5}$).

Finally, we investigated the potential biological roles of the resulting list of genes in brain disorders. For this, we performed functional analysis with FUMA GWAS[21]. We summarized the independent genomic risk loci from

SNP	CHR:BP	Allele (min/maj)	MAF (1KG)	MAF Cohort 1	MAF Cohort 2	Gene	Meta Stage 1		Meta Stage 2	
							OR	P	OR	P
rs429358	19:45411941	C/T	0.155	0.143	0.153	*APOE e4*	2.79	2.00e-14	2.28	6.15e-17
rs12734374	1:155388851	T/A	0.023	0.022	0.012	*ASH1L/GBA*	4.29	4.29e-09	4.31	1.33e-09
rs12926163	16:88572056	C/T	0.311	0.252	0.323	*ZFPM1*	1.68	1.45e-07	—	—

Table 1. Genetic loci with significant and suggestive associations with DLB at meta-analysis Stage 1 and Stage 2. CHR:BP: Chromosome and Base pair location based on Build 37, Assembly Hg19. Allele (min/maj): Minor and major alleles; MAF: Minor Allele Frequency (on European 1000 G), Cohort 1 and Cohort 2. Gene: Nearest gene within 500 kB; OR, P: case-control odds-ratio and association P-values from Stage 1 combining Cohorts 1 and 2, and Stage 2 Meta-analysis combining Cohorts 1, 2 and 3.

Stage 1 with suggestive association $p < 1 \times 10^{-6}$ in S. Fig. 6. The strongest associations were close to *APOE* in Chromosome 19 and distributed in a relatively small region spanning only 41kB and 4 genes. The significant associations in Chromosome 1, within the large haploblock containing *GBA*, spanned 1.2MB and up to 64 genes (see S. Fig. 6). We computed gene-based P-value test for protein-coding genes by mapping SNPs to genes if SNPs were located within the genes. The chromosome 1 genes (*GBA*) did not surpass the significance threshold (S. Fig. 7), but the chromosome 19 genes (*APOE, APOC1, TOMM40*) remained significant. We also found significant results applying the gene-based test to the gene on chromosome 16 (*ZPFM1*), which was suggestive at the single variant level. Using MAGMA tissue expression analysis, we found the strongest expression in whole blood, substantia nigra and spinal cord cervical level c-1. (S. Fig. 8).

FUMA prioritized 65 genes (S. Table 3, S. Fig. 9) for further functional analyses; see Methods on how genes are prioritized. Of note, in Chromosome 1, *GBA* was prioritized based on eQTL analysis (S. Fig. 9), further strengthening the case that our top hit implicated not only *ASH1L* but also *GBA*. From the set of 65 genes, we looked up tissue specific expression patterns based on GTEx v6 RNA-seq data. These are visualized as a heatmap in S. Fig. 10[21]. Relative to other genes, we found *APOE* highly expressed in all tissues (S. Fig. 10A). *ASH1L* and *GBA* are moderately expressed and *ZFPM1* has a lower gene expression relative to other genes in all tissues. Next, we looked at the tissue specificity for each gene. We found *APOE* with moderately higher expression in brain tissue (S. Fig. 10B), while *ASH1L*, *GBA* and *ZFPM1* are not specific to brain tissues. Notably, we found higher expression in brain tissues for *PAQR6*, *CHRNB2*, *SYT11* and *APOC1* and conversely we found lower expression for *PVRL2*, *LMNA* and *SHC1* (S. Fig. 10B).

Besides the single gene level analyses, we also identified tissue specificity of prioritized genes by looking at overrepresentation in sets of differentially expressed genes (DEG), see S. Fig. 11. DEG for each tissue was calculated in FUMA. We found the spinal cord cervical level c-1 and amygdala being two of the top five tissues with the most DEG, however, none passed Bonferroni corrected significance. The finding of the spinal cord cervical level c-1 is consistent with the MAGMA analysis.

Discussion

We performed a genome-wide association study based on 828 clinically diagnosed DLB cases and a large sample of 82035 controls. We confirmed the *APOE ε4* allele and a locus close to *ASH1L* and *GBA* (Chr1q22) as significantly associated with DLB. Furthermore, we nominate a novel genetic locus near *ZFPM1* as suggestively associated with DLB. Taken together with recent findings from another DLB GWAS[13], the current results firmly establish *APOE e4*, *SNCA* and *GBA* as robust risk loci for DLB, which implicate novel disease mechanisms to be followed up in experimental studies.

The top-hit SNP at the 1q22 locus is located within the large haploblock containing the *GBA* (glucocerebrosidase gene), also recently identified by Guerreiro *et al.*[13]. We note that the strongest association was in SNPs with relatively high LD (D′ 0.66) with rs2230288, referred to in the literature as the *GBA* E326K or 365 K polymorphism. E326K is a low frequency coding variant, which unlike the "neuropathic" *GBA* mutations does not cause Gaucher's disease in the homozygous state. We recently demonstrated that this variant accounts for the *GBA* top-hit from a PD meta-GWAS[23]. E326K has also been associated with worse cognitive outcomes in PD[15,16]. We inspected the association results further and found that E326K showed the strongest of all associations at this locus in Cohort 1, yet was not successfully imputed in Cohort 2. We thus consider it likely that E326K is the functional variant underlying this signal. With an allele frequency of >2% in the population and a strong effect on susceptibility to both PD and DLB, this variant emerges as a major risk factor for the Lewy body disorders combined. From a functional perspective, the *GBA* association highlights the importance of lysosomal pathways in DLB pathogenesis. *GBA* was recently confirmed in the largest GWAS in DLB to date (1743 DLB patients included) as the third most strongly associated risk gene[13]. *SCARB2 (scavenger receptor class B member 2)*, encoding another lysosomal enzyme, has previously been associated with DLB[12]. While *GBA* is probably the most plausible causative gene in the 1q22 locus, we cannot rule out other candidate gene such as *ASH1L* (Absent, Small or Homeotic discs 1-Like). The gene encodes a histone-lysine N-methyltransferase, a member of the trithorax transcriptional regulators which are essential for development, organ function and fertility.

We and others have previously reported *APOE (Apolipoprotein E) ε4* (Chr19q13.32) as an important genetic risk factor for DLB. Guerreiro *et al.* found the locus highly significant[13], and we reported an OR for carriers of one copy of the *APOE ε4* allele to be 2.9 for developing DLB and 4.2 for developing AD. For carriers of two copies of the *APOE ε4* alleles the OR for developing DLB increased to 5.9 while the OR for developing AD was as high as 15.2[14]. Bras and colleagues have reported *APOE* as the strongest associated risk gene in both clinically

and neuropathologically diagnosed DLB cases[12], and this was confirmed in an expanded cohort from the same group recently[13]. Guerreiro *et al.* estimated the DLB SNP-heritability based on the Illumina Neuro-X content to be 31%, with *APOE* accounting for about 9%[11]. *APOE ε4* has also been found to increase the risk of dementia in pure alpha-synucleinopathies in a study where its frequency was 38% in pathologically diagnosed pure AD, 40.6% in the mixed AD and DLB group, 31.9% in pure DLB, 19.1% in Parkinson's Disease Dementia (PDD) and 7.2% among healthy controls[24]. In another AD GWAS, Lewy body pathology in the brain was associated with *APOE* variants[25]. Most cases with clinically diagnosed DLB will contain both Lewy bodies and AD pathology in the brain[7].

SNCA (synuclein alpha) is the strongest associated risk gene in PD and encodes α-synuclein, which is a major constituent of Lewy bodies, pathological hallmark for both DLB and PD/PDD. Accumulation of α-synuclein aggregates have been found to create synaptic dysfunction in DLB[26]. The top associated variant in our data (rs2301135) is in LD with the *SNCA* signal reported as significant in the previous study by Bras *et al.* (r-squared 0.98 and D′ 1.0 with rs894280 in 1000 genomes European population)[12] and the secondary signal from a large meta-analysis of PD GWAS (r-squared 0.98 and D′ 1.0 with rs7681154)[27]. Both p-value and effect size of the *SNCA* association observed here are equivalent to those found in the similarly sized DLB study by Bras *et al.*[12], and despite falling short of genome wide significance, we interpret this result as supportive for an *SNCA* association in DLB. Deviations from other studies with respect to the strongest SNP at the locus could well arise if key SNPs are not well imputed across all cohorts.

Together, the identified genetic loci could be involved in a common neurobiological disease pathway in DLB. The normal degradation of α-synuclein is highly dependent on lysosomal function and glucocerebrosidase is an important enzyme in this degradation. Impaired function of glucocerebrosidase due to coding variants like E326K will slow down the degradation of α-synuclein thus increasing the concentration of toxic oligomers and thereby driving the pathological process in DLB. Inhibition of lysosomal enzymes also results in Aβ accumulation and aggregation. The apolipoproteins accelerate neuronal Aβ uptake, lysosomal trafficking and degradation in an isoform-dependent manner with apolipoprotein E3 more efficiently facilitating Aβ trafficking and degradation than apolipoprotein E4, a risk factor for AD and DLB[28], thus linking both *GBA*, *APOE* and *SNCA*.

The present findings of genetic loci suggestively associated with DLB indicate interesting pathological mechanisms. The chromosome 16 locus associated with DLB at $p = 1.45 \times 10^{-7}$ implicates *ZFPM1* (Zinc finger protein, FOG family member 1), which is expressed in human hematopoietic tissues and in the cerebellum and is involved in erythroid differentiation. In one study of AD and psychosis, duplications in this gene were associated with psychosis in AD, a symptom highly relevant in DLB, were visual hallucinations and related delusions are core symptoms of the disease[29]. Our findings suggest other putative molecular mechanisms in DLB.

CNTN1 (contactin 1) is a glycosylphosphatidylinositol anchored neuronal membrane protein that functions as a cell-adhesion molecule with important roles in axonal function. It is located near the *LRKK2* locus and was associated to PD and reported as a suggestive hit in the largest GWAS of DLB to date[13]. We found no significant hits under correction in our study. Further, we found no genome-wide significant associations with *MAPT (microtubule associated protein tau)*, the gene encoding tau, in our study. However, this gene was poorly covered in our genotyping and imputation procedure. *MAPT* is the second strongest associated risk gene in PD and is associated also with AD[30]. It exists on two different haplotypes, H1 and H2. H1P has been associated with Parkinson's disease with dementia, whereas H1C has been associated with Alzheimer's disease. Thus, *MAPT* would be a plausible candidate gene also for DLB due to clinical and likely genetic overlap with AD and PD beyond *APOE*[31,32]. The negative finding suggests that the role of *MAPT* variability might represent a genetic difference between DLB and PD, but this hypothesis needs to be further tested in larger cohorts, preferentially including both DLB and PD samples.

All cases included in our study were clinically diagnosed with common sporadic and late onset type of DLB. Cases solely diagnosed based on pathology might not always fulfil clinical diagnostic criteria during life, and therefore might include cases of PD and even early onset PD developing dementia in late stages. The clinically diagnosed DLB cases included in our study might therefore have a purer, less PD-like genetic profile than studies based on brain bank cases, a possible explanation for why we do not find the previously PD-associated risk loci like *MAPT* in our analysis. Diagnosing DLB clinically is challenging both because of the clinical heterogeneity and the overlapping AD pathology masking typical DLB core symptoms in many cases. Although diagnostic procedures differed among centres, nearly all centres are academic dementia research centres with high-level clinical expertise on DLB and used standardized and established procedures, including, in a subset of patients, biomarkers. Thus, we believe diagnoses were as accurate as can be achieved in a clinical setting, although pathological confirmation was available only in a subset. The clinical diagnostic criteria for probable DLB have been found to have high specificity and this was confirmed in a pathological study in one of the samples included in this study[7]. We therefore argue that only few cases with other diagnoses like AD can have been erroneously included in this sample. Adding biomarkers like (123)-FP-CIT-SPECT to the diagnostic procedure could increase diagnostic precision in DLB. AD-pathology could be detected by PET or CSF-biomarkers of amyloid and tau deposition[33]. The recently published revised diagnostic criteria for DLB are slightly different from the previous[1]. Whether this will impact on the genetic architecture of DLB cohorts is not known, however.

There are few other large cohorts diagnosed with DLB with well characterized patients, and although this is one of the largest studies in DLB to date – sample size is still small for a hypothesis-free GWAS approach. Consequently, we only had statistical power to detect signals with large effect sizes, such as *APOE* and *GBA*. We anticipate that GWAS with larger samples will detect more common genetic risk loci associated with DLB with effect sizes comparable to the vast majority of AD and PD GWAS loci. Current evidence further indicates that rare variants contribute significantly to the disorder, suggesting next generation sequencing approaches will also be important to further characterize the genetic architecture of DLB.

DLB is increasingly recognized as a specific clinical diagnosis distinct from AD and PD both clinically and genetically, and has a poor prognosis with no approved treatment. To detect more of the genetic risk loci contributing to DLB pathogenesis new methods like Bayesian statistics may prove useful. This notwithstanding, larger samples obtainable through international collaboration are needed in a future GWAS of DLB. We therefore plan to collaborate to increase sample size in a next step to increase the power to detect more common genetic variants with small effects associated with the risk of development of DLB.

Data Availability

Genotype datasets from the Norwegian DemGene network generated and analysed during the current study are not publicly available due to compliance to privacy. Summary statistics are available from the corresponding author on reasonable request.

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Author Contributions

Arvid Rongve collected samples, initiated and planned the study, organized recruitment of cohorts, wrote the first draft and updated the manuscript based on input from all co-authors. Aree Witoelar conducted biostatistical analyses in Cohort 1 and combined analyses, and wrote the manuscript. Dag Aarsland and Ole A. Andreassen organized and planned the study, coordinated and supervised the work, and wrote the manuscript. Agustín Ruiz did the biostatistical analyzes in Cohort 2 and helped with writing the manuscript. Francesco Bettella and Jon A. Eriksen were involved in genotype quality control and parts of biostatistical analyses. Lavinia Athanasiu was involved in genotyping and data collection. Kári Stefánsson and Hreinn Stefánsson genotyped Cohort 1 and provided Cohort 3 samples. Stefanie Heilmann-Heimbach did the genotyping of Cohort 2. Lasse Pihlstrøm collected samples and contributed to writing the manuscript. Carla Abdelnour and Jordi Clarimon, Isabel Hernández, Sonia Moreno-Grau, Itziar de Rojas, Estrella Morenas-Rodríguez, Sigrid B. Sando, Geir Bråthen, Frédéric Blanc, Olivier Bousiges, Afina W. Lemstra, Inger van Steenoven, Elisabet Londos, Ina S. Almdahl, Lene Pålhaugen, Srdjan Djurovic, Eystein Stordal, Ingvild Saltvedt, Ingun D. Ulstein, Ane-Victoria Idland, Mathias Toft, Jon Snaedal, Lluís Tárraga, Mercè Boada, Alberto Lleó and Alfredo Ramírez all organized and collected samples at different study sites. Tormod Fladby planned the study and coordinated Norwegian sites, and together with Rahul Desikan provided input to interpretation of the results. All co-authors read and approved the paper.

Additional Information

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Annex 5

Article: CSF progranulin increases in the course of Alzheimer's disease and is associated with sTREM2, neurodegeneration and cognitive decline

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Research Article

TRANSPARENT PROCESS OPEN ACCESS

EMBO Molecular Medicine

CSF progranulin increases in the course of Alzheimer's disease and is associated with sTREM2, neurodegeneration and cognitive decline

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Abstract

Progranulin (PGRN) is predominantly expressed by microglia in the brain, and genetic and experimental evidence suggests a critical role in Alzheimer's disease (AD). We asked whether PGRN expression is changed in a disease severity-specific manner in AD. We measured PGRN in cerebrospinal fluid (CSF) in two of the best-characterized AD patient cohorts, namely the Dominant Inherited Alzheimer's Disease Network (DIAN) and the Alzheimer's Disease Neuroimaging Initiative (ADNI). In carriers of AD causing dominant mutations, cross-sectionally assessed CSF PGRN increased over the course of the disease and significantly differed from non-carriers 10 years before the expected symptom onset. In late-onset AD, higher CSF PGRN was associated with more advanced disease stages and cognitive impairment. Higher CSF PGRN was associated with higher CSF soluble TREM2 (triggering receptor expressed on myeloid cells 2) only when there was underlying pathology, but not in controls. In conclusion, we demonstrate that, although CSF PGRN is not a diagnostic biomarker for AD, it may together with sTREM2 reflect microglial activation during the disease.

Keywords Alzheimer's disease; biomarker; microglia; progranulin; TREM2
Subject Categories Biomarkers & Diagnostic Imaging; Neuroscience
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Introduction

Haploinsufficiency of the gene encoding progranulin (PGRN) leads to frontotemporal lobar degeneration (FTLD) with TAR DNA binding protein 43 (TDP-43) deposition (FTLD-TDP) (Baker *et al*, 2006; Cruts *et al*, 2006; Neumann *et al*, 2006). Reduced PGRN protein in plasma, serum or cerebrospinal fluid (CSF) has been shown to be a reliable diagnostic biomarker for early detection of *progranulin* (*GRN*) mutation carriers (Ghidoni *et al*, 2008; Finch *et al*, 2009; Sleegers *et al*, 2009). Certain *GRN* variants may also increase the risk for Alzheimer's disease (AD) (Brouwers *et al*, 2008; Rademakers *et al*, 2008; Viswanathan *et al*, 2009; Lee *et al*, 2011; Cruchaga *et al*, 2012; Sheng *et al*, 2014; Xu *et al*, 2017), yet associations could not be confirmed in some other studies (Fenoglio *et al*, 2009; Mateo *et al*, 2013). Studies in AD mouse models indicated that PGRN is strongly increased in microglia clustering around amyloid plaques (Pereson *et al*, 2009) and may also affect amyloid β-peptide (Aβ) and tau deposition. PGRN may have beneficial effects on Aβ deposition, since PGRN deficiency in animal models increases Aβ deposition (Minami *et al*, 2014) and elevating PGRN expression reduces the amyloid plaque burden (Minami *et al*, 2014; Van Kampen & Kay, 2017). However, it has also been observed that PGRN deficiency leads to a decrease in diffuse Aβ plaque load (Takahashi *et al*, 2017; Hosokawa *et al*, 2018), which may point to a detrimental effect of PGRN in Aβ deposition. In regard to tau pathology, PGRN deficiency accelerates tau deposition and phosphorylation in human tau-expressing mice (Hosokawa *et al*, 2015; Takahashi *et al*, 2017). Furthermore, an AD-associated *GRN* variant (*rs5848*), which causes a decrease in PGRN levels in plasma and CSF (Rademakers *et al*, 2008; Nicholson *et al*, 2014; Morenas-Rodríguez *et al*, 2015), is associated with increased CSF T-tau levels in participants of the ADNI study (Takahashi *et al*, 2017). Together, these results indicate a protective role of PGRN against the development of tau pathology and/or neurodegeneration.

PGRN is a 593 amino acid protein that contains seven and a half tandem repeats forming the granulin domains (Bateman *et al*, 1990; Bhandari *et al*, 1992). It is targeted through the secretory pathway and secreted into the extracellular space. Secreted PGRN can be taken up and targeted to endosomes/lysosomes (Hu *et al*, 2010; Zhou *et al*, 2015b). Proteolytic processing of full-length PGRN may generate individual granulin peptides (Kleinberger *et al*, 2013; Holler *et al*, 2017). Within the brain, PGRN is predominantly expressed by microglia (Zhang *et al*, 2014; Lui *et al*, 2016; Chang *et al*, 2017), but some expression is also observed in neurons. Moreover, PGRN is significantly increased upon microglial activation (Daniel *et al*, 2000; Naphade *et al*, 2010; Petkau *et al*, 2010; Philips *et al*, 2010; Kleinberger *et al*, 2013; Suh *et al*, 2014). PGRN may be involved in the modulation of neuroinflammation since PGRN-deficient mice exhibit increased microglial activation and astrogliosis, as well as augmented expression of proinflammatory cytokines (Yin *et al*, 2009, 2010; Ahmed *et al*, 2010; Martens *et al*, 2012; Wils *et al*, 2012; Filiano *et al*, 2013; Minami *et al*, 2014). Whereas deficiency in the triggering receptor expressed on myeloid cells 2 (TREM2) locks microglia in a homeostatic stage (Mazaheri *et al*, 2017), loss of PGRN leads to their hyperactivation. Thus, deficiency of PGRN and TREM2 results in opposite functional deficits (Götzl *et al*, submitted).

Although genetic data and functional analyses in animal models provide some evidence for an involvement of PGRN in AD, no changes in CSF PGRN in AD compared to healthy controls have been reported so far (Nicholson *et al*, 2014; Körtvélyessy *et al*, 2015; Morenas-Rodríguez *et al*, 2015; Wilke *et al*, 2017). However, these studies focused primarily on AD dementia and did not investigate the entire *continuum* of AD. Given that CSF biomarkers, such as the shed ectodomain of the triggering receptor expressed on myeloid cells 2 (sTREM2), show dynamic changes throughout the course of AD (Suárez-Calvet *et al*, 2016a,b), we aimed to cross-sectionally assess CSF PGRN changes at different stages of the disease. To this end, we studied a sample of persons at-risk for autosomal dominant AD (ADAD) recruited within the Dominantly Inherited AD Network (DIAN; http://dian.wustl.edu) (Bateman *et al*, 2012) and participants with late-onset AD from the Alzheimer's Disease Neuroimaging Initiative (ADNI; http://adni.loni.usc.edu; Weiner *et al*, 2012). We investigated whether CSF PGRN (i) increases in relation to the clinical course of AD; (ii) is associated with cognitive impairment and neuroimaging markers of neurodegeneration (fludeoxyglucose positron emission tomography, FDG-PET and hippocampal volume) in subjects with AD; and (iii) is associated with the microglial-derived protein sTREM2 as well as biomarkers of amyloid and tau pathology.

Results

CSF PGRN increases throughout the course of autosomal dominant Alzheimer's disease

Cross-sectionally, we assessed CSF PGRN in 215 participants from the DIAN initiative, including 130 mutation carriers (MC) and 85 non-carriers (NC; Table 1). All analyses described were adjusted for gender, age and *APOE* ε4 status, unless stated otherwise.

The levels of CSF PGRN were significantly increased in MC compared to NC ($F_{1,210} = 17.6$, $P = 0.00004$, Fig 1A, Appendix Table S1). In contrast to CSF sTREM2 (Henjum *et al*, 2016; Heslegrave *et al*, 2016; Piccio *et al*, 2016; Suárez-Calvet *et al*, 2016a,b), CSF PGRN was not significantly associated with age, neither in the entire sample ($\beta = 0.118$, $P = 0.079$), nor when stratifying by mutation status (for NC: $\beta = 0.125$, $P = 0.283$; for MC: $\beta = 0.131$, $P = 0.142$, Fig 1B). Consistent with previous publications (Nicholson *et al*, 2014; Morenas-Rodríguez *et al*, 2015), CSF PGRN was higher in males than in females ($F_{1,210} = 6.35$, $P = 0.012$, Fig 1C) and were not affected by *APOE* ε4 status ($F_{1,210} = 0.041$, $P = 0.840$). CSF PGRN did not differ between the three ADAD-associated genes (*PSEN1*, *PSEN2* and *APP*) among the MC participants ($F_{2,124} = 0.77$, $P = 0.464$, Fig 1D).

We determined how CSF PGRN changes as a function of the estimated years from expected symptom onset (EYO) in MC compared to NC. Mean estimated levels of CSF PGRN at consecutive 5-year interval EYO were computed by a linear regression model including mutation status, EYO and gender as predictor variables (see Statistical analysis section). As described by Bateman *et al* (2012), we tested first-, second- and third-order EYO terms (EYO, EYO^2 and EYO^3, respectively) as well as their interaction with mutation status. The first-order (i.e. linear) model best fitted the data (Appendix Table S2). The interaction of EYO with mutation status

Table 1. DIAN participants' characteristics.

	Autosomal dominant Alzheimer's disease (DIAN study)		
	Non-carriers (NC) (n = 85)	Mutation carriers (MC) (n = 130)	P-value (group effect)
Age, years	40.0 (10.8)	39.9 (10.7)	0.955
Females, %	55.3	49.2	0.384
APOE ε4 carriers, %	35.3	27.7	0.237
Participant EYO, y	−6.81 (11.7)	−6.68 (10.7)	0.931
Education level, y	14.8 (2.40)	13.7 (3.19)	0.005*
MMSE, scores	29.0 (1.26)	25.6 (6.16)	< 0.0001*
CSF biomarkers, pg/ml[a]			
T-tau	60.1 (28.1)	130 (97.0)	< 0.0001*
P-tau$_{181P}$	30.5 (10.3)	69.8 (42.0)	< 0.0001*
Aβ$_{1–42}$	425 (136)	322 (160)	< 0.0001*
sTREM2	2,796 (1,305)	3,520 (1,640)	0.0007*
PGRN[b]	927 (174)	1,041 (199)	0.00004*
Cognitive status, %[c]			
CDR = 0	96.5	43.1	
CDR = 0.5	3.5	39.2	
CDR = 1	0	11.5	
CDR = 2–3	0	6.1	
Family mutations, %[d]			
PSEN1	58.8	77.7	
PSEN2	14.1	8.5	
APP	27.1	13.8	

Aβ$_{1–42}$, amyloid-β 42; AD, Alzheimer disease; *APOE*, apolipoprotein E; APP, amyloid precursor protein; CSF, cerebrospinal fluid; EYO, estimated years from expected symptom onset; MMSE, Mini-Mental State Examination; *PSEN1*, presenilin 1; *PSEN2*, presenilin 2; P-tau$_{181P}$, tau phosphorylated at threonine 181; T-tau, total tau; y, years.
Data are expressed as mean (M) and standard deviation (SD) or percentage (%), as appropriate. Pearson's chi-square tests were used for the group comparisons of categorical variables and two-sample independent t-tests to compare continuous variables.
*Significant differences. The P-values indicated in the last column refer to the group effects in these tests.
[a]CSF core biomarkers in DIAN were measured using the Luminex bead-based multiplexed xMAP technology (INNO-BIA AlzBio3, Innogenetics).
[b]CSF PGRN differences were assessed by a linear model adjusted for age, gender and *APOE* ε4 status (see main text).
[c]Cognitive status was defined by the clinical dementia rating (CDR) score (0, cognitively normal; 0.5, very mild; 1, mild; 2, moderate; 3, severe dementia).
[d]In NC participants, the mutation present in their family is shown.

was a significant predictor of CSF PGRN changes (P = 0.041), indicating that the changes in CSF PGRN as a function of EYO differ between MC and NC. We compared the estimated CSF PGRN at 5-year EYO intervals by t-tests and observed that CSF PGRN started to be significantly increased in MC compared to NC at EYO = −10 (Table 2, Fig 2A, Appendix Table S3).

Following the approach of previous DIAN studies (Bateman *et al*, 2012; Fagan *et al*, 2014; Suárez-Calvet *et al*, 2016a,b), we added CSF PGRN in the temporal sequence of biomarker changes within the course of ADAD (Fig 2B). Like CSF sTREM2, changes in CSF PGRN occurred after alterations in markers for brain amyloidosis and neuronal injury, as measured by CSF T-tau.

Next, we compared CSF PGRN in MC at different clinical stages defined by the clinical dementia rating (CDR) score with that of the NC. We hence compared four groups: NC, cognitively normal (CDR = 0) MC, very mild dementia (CDR = 0.5) MC and mild-to-severe dementia (CDR > 1) MC. Participants with a CDR ≥ 1 were grouped together because of the low number of subjects in these groups (Table 1). We conducted an analysis of covariance (ANCOVA) adjusted for age, gender, *APOE* ε4 status and education, and we observed a significant difference between the four groups ($F_{3,207}$ = 5.77, P = 0.001). A least significant difference (LSD) pairwise *post hoc* comparisons revealed that all MC groups (CDR = 0: M = 1002, SD = 186; CDR = 0.5: M = 1072, SD = 201; CDR ≥ 1: M = 1065, SD = 214; pg/ml) had significantly higher CSF PGRN than the NC (M = 927 pg/ml, SD = 174; P = 0.026, P = 0.0001 and P = 0.018, respectively). No differences were found between the MC groups at different clinical stages. Likewise, CSF PGRN levels were not associated with Mini-Mental State Examination (MMSE; P = 0.881) or CDR sum of boxes (CDR-SB; P = 0.812) among MC.

CSF PGRN increases throughout the course of late-onset Alzheimer's disease

To further validate our findings in subjects with late-onset AD, we studied a total of 1,017 participants of the ADNI study (demographics of the entire ADNI sample are summarized in Appendix Table S4). In the entire sample, and consistent with the

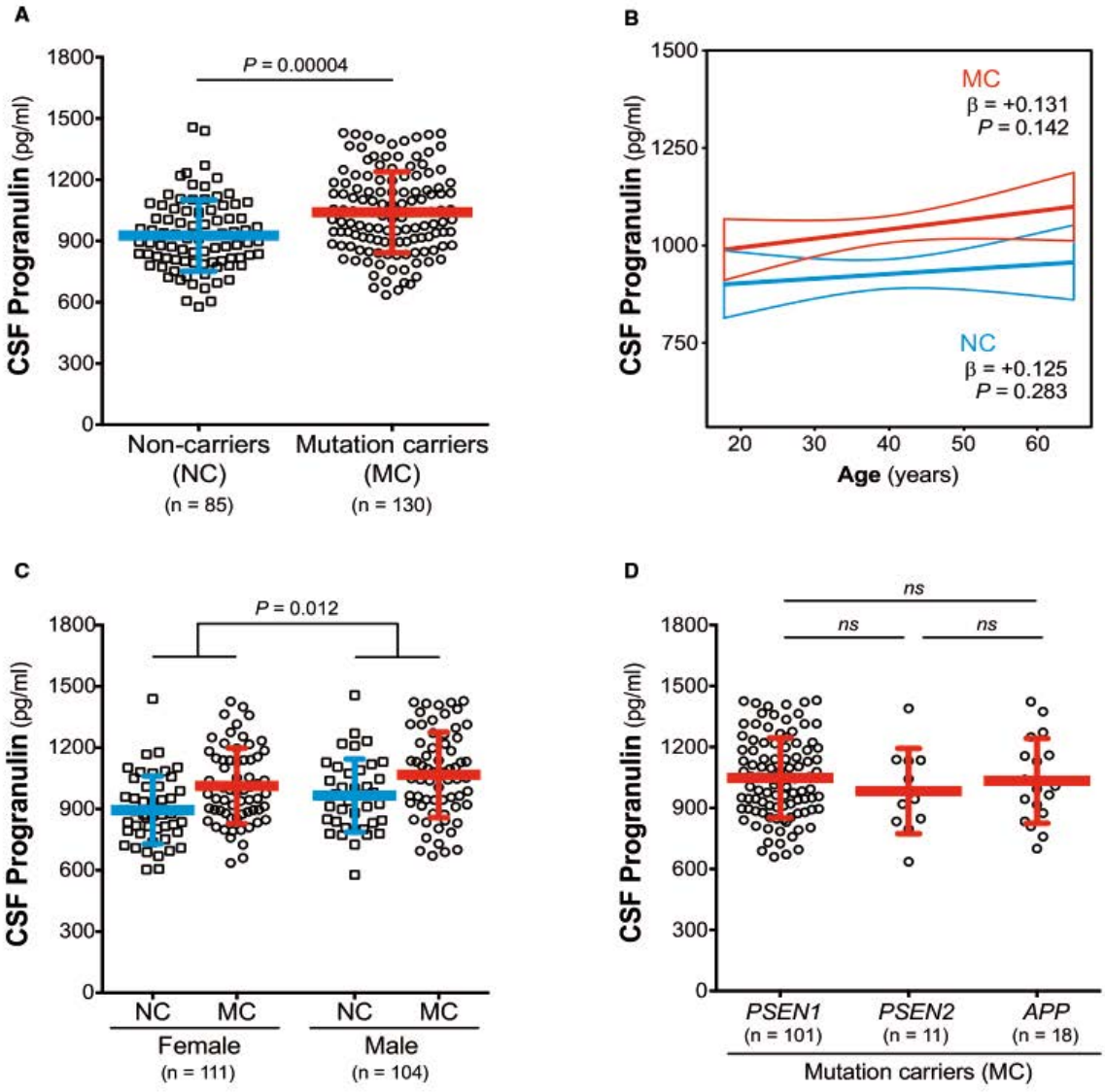

Figure 1. Association of CSF PGRN with mutation status, age and gender.

A CSF PGRN is increased in mutation carriers (MC) compared to non-carriers (NC).

B CSF PGRN is not associated with age in either NC or MC.

C CSF PGRN is increased in males compared to females.

D CSF PGRN levels do not differ among MC participants carrying a *PSEN1*, *PSEN2* or *APP* mutation.

Data information: The blue or red bars in (A), (C) and (D) represent the mean and the standard deviation (SD). Group comparisons were assessed by a linear model adjusting by age, gender and *APOE* ε4 status. The solid lines in (B) indicate the regression line for each of the groups and the 95% confidence interval (CI) calculated by a linear model adjusting by gender and *APOE* ε4 status. The standardized regression coefficients (β) and the *P*-values are also shown. In graph (B), the individual values are not shown in order to protect participants' confidentiality. All analysis and graphs are performed excluding 3 PGRN values outliers. Including the outliers in the analysis rendered similar results (Appendix Table S1). *APP*, amyloid precursor protein; CSF, cerebrospinal fluid; ns, non-significant; *PSEN1*, presenilin 1; *PSEN2*, presenilin 2.

Table 2. CSF PGRN estimates (pg/ml) in MCs and NCs as a function of EYO.

	Estimated years from expected symptom onset (EYO)							
	−25	−20	−15	−10	−5	0	+5	+10
Non-carriers	974	978	983	988	992	997	1,002	1,006
Mutation carriers	992	1,022	1,052	1,082	1,112	1,142	1,172	1,202
Difference	18	44	69	94	120	145	170	196
95% CI	[−104, 140]	[−64, 151]	[−27, 165]	[4, 184]	[29, 210]	[48, 242]	[62, 279]	[72, 319]
P-value	0.768	0.423	0.159	0.040*	0.010*	0.004*	0.002*	0.002*

CI, confidence interval.
Mean estimated levels of CSF PGRN were obtained by a linear model including gender, mutation status, EYO and the interaction between mutation status and EYO as covariates (see Statistical analysis section and Appendix Table S2). For each EYO, the group difference, 95% CI and the *P*-value for the two-sample independent *t*-test are reported. Participants with EYO > +20 (1 NC and 2 MC) were excluded from the analysis. The same analysis including the PGRN outliers and those participants with an EYO > +20 yielded identical results (Appendix Table S3). Differences are calculated from unrounded values.
*Significant difference.

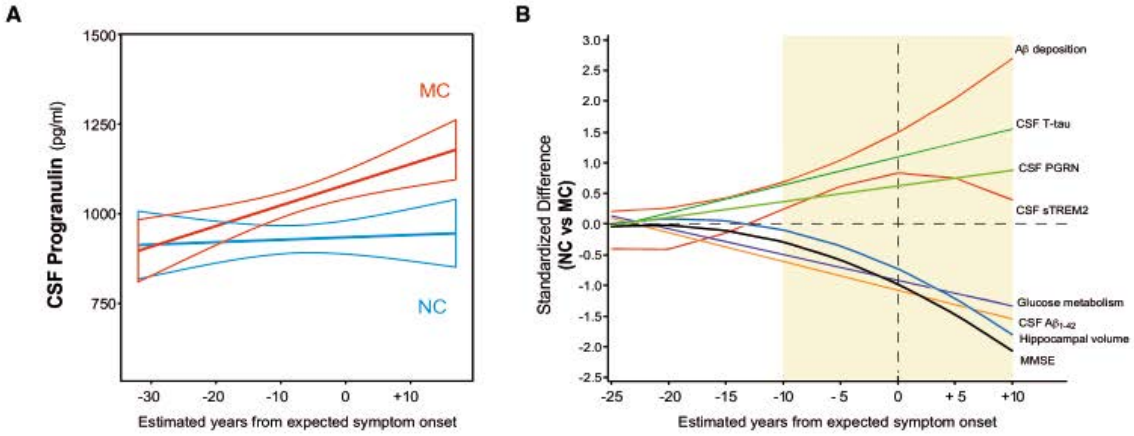

Figure 2. Changes in CSF PGRN as a function of EYO.

A CSF PGRN as a function of EYO in mutation carriers (MC, red) and non-carriers (NC, blue). The solid lines indicate the regression line for each of the groups and the 95% confidence interval (CI) calculated by a linear model adjusting by gender. The interaction term of mutation status and EYO is significant (P = 0.041), also when including PGRN outliers and participants with EYO > +20 (P = 0.030). Individual data points are not displayed to prevent disclosure of mutation status.

B The graph depicts the standardized differences in CSF PGRN between MCs and NCs as a function of EYO, in the context of other biomarker and cognitive changes. The curves were generated by the linear model that best fit each marker (see Statistical analysis section and Appendix Table S2). CSF PGRN is significantly increased in MC compared to NC 10 years before the expected symptom onset (shadowed area) after brain amyloidosis and brain injury (as measured by CSF T-tau) have started, and shortly before CSF sTREM2 starts to increase.

Data information: $A\beta_{1-42}$: amyloid-β 42; CSF, cerebrospinal fluid; MC, mutation carrier; MMSE, Mini-Mental State Examination; NC, non-carrier; T-tau, total tau.

findings in ADAD, CSF PGRN was higher in males than females ($F_{1,1013}$ = 26.4, P < 0.0001, Appendix Table S5). A trend towards lower PGRN was observed in *APOE* ε4 carriers ($F_{1,1013}$ = 3.40, P = 0.066), but was not associated with age (β = +0.049, P = 0.118).

In order to study the changes in CSF PGRN at different stages of disease severity, we defined disease stage according to the recently proposed biomarker-based A/T/N framework (Jack *et al*, 2016a) in combination with the CDR score (Morris, 1993; Table 3). The A/T/N classification is the basis of the 2018 National Institute on Aging–Alzheimer's Association (NIA-AA) Research Framework (Jack *et al*, 2018) and defines three binary biomarker categories: (i) aggregated Aβ (A+/A−), (ii) aggregated tau (T+/T−) and (iii) neurodegeneration or neuronal injury (N+/N−). Herein, each of these categories was defined using the AD CSF core biomarkers, namely CSF $A\beta_{1-42}$ (A), P-tau_{181P} (T) and T-tau (N). The aggregated tau (T) and neurodegeneration (N) groups were merged together to simplify the number of groups to compare (only 5.3% of the participants of the ADNI total sample displayed discrepancies between the T and N biomarker groups; see Materials and Methods section for a comprehensive description on the classification). We also classified the participants based on their clinical status (C), as measured by the well-established CDR global score (Morris, 1993), into cognitively unimpaired (CDR = 0), very mild dementia (CDR = 0.5) and mild dementia (CDR = 1). The combination of both the biomarker

Table 3. Classification of ADNI participants based on their biomarker profile and clinical stage.

		Clinical stage (C)		
		CDR = 0 (cognitively unimpaired)	CDR = 0.5 (very mild dementia)	CDR = 1 (mild dementia)
Biomarker profile	A−/TN−	**Healthy controls** n = 128	n = 120	n = 2
	A+/TN−	**Preclinical AD A+/TN−** n = 56	n = 96	n = 15
	A+/TN+	**Preclinical AD A+/TN+** n = 48	**AD CDR = 0.5** n = 289	**AD CDR = 1** n = 81
	A−/TN+	n = 74	n = 100	n = 8
		suspected non-Alzheimer's pathophysiology (SNAP)		

A, amyloid-β biomarker status; AD, Alzheimer's disease; CDR, clinical dementia rating; N, neurodegeneration biomarker status; T, tau pathology biomarker status.
ADNI participants were classified based on their CSF biomarker profile and their clinical stage, which yielded 12 different categories. Columns depict the clinical stage (C) as defined by the clinical dementia rating (CDR) scale. Rows depict the biomarker profiles. Each of the three biomarker groups (A/T/N) was binarized into positive or negative (+/−). T and N were merged to simplify the classification: TN− indicates that both T and N biomarkers are normal, and TN+ indicates that T and/or N biomarkers are abnormal.
The grey highlighting indicates the grouping used for comparisons in the main text. Light grey highlights the healthy controls (n = 128), middle grey the groups included in the Alzheimer's *continuum* (n = 474) and dark grey the suspected non-Alzheimer's pathophysiology (SNAP) group (n = 182). Bold text indicates the groups analysed in the main analysis, namely "healthy controls", "Preclinical AD A+/TN−", "Preclinical AD A+/TN+", "AD CDR = 0.5" and "AD CDR = 1".

and the clinical classification rendered twelve different groups that are summarized in Table 3. After classifying the participants of the ADNI sample, we analysed the data following two approaches. In a first approach, we attempted to model disease stages within the Alzheimer's *continuum* as a combination of biomarkers and clinical symptoms similar to what was proposed by the previous 2011 NIA-AA diagnostic criteria (Albert *et al*, 2011; McKhann *et al*, 2011; Sperling *et al*, 2011), and it was done in order to render the disease staging in late-onset AD more comparable to that in ADAD defined by EYO. Second, in an exploratory approach, we compared PGRN levels between the different A/T/N categories within each clinical stage.

Thus, we first asked whether CSF PGRN increases in relation to the biomarker-defined clinical stages, and hence parallels PGRN changes as a function of EYO in ADAD. For this purpose, we compared the "healthy control" group (highlighted in light grey in Table 3) with those groups that belong to the Alzheimer's *continuum* (highlighted in middle grey in Table 3). We hence compared five groups in this first analysis, namely (i) "healthy controls", (ii) "Preclinical AD A+/TN−" (iii) "Preclinical AD A+/TN+" (iv) "AD CDR = 0.5" and (v) "AD CDR = 1".

The demographics and clinical features of these groups are summarized in Table 4. We conducted an ANCOVA controlling for age, gender and *APOE* ε4 status, and we found that CSF PGRN significantly differed between groups ($F_{4,594}$ = 7.32, P < 0.0001). Bonferroni corrected pair-wise *post hoc* comparisons indicated that CSF PGRN was significantly higher in the "AD CDR = 1" group compared to the "healthy controls" (P = 0.006) and "Preclinical AD A+/TN−" (P < 0.0001) groups (Fig 3 and Table 4). Interestingly, the "Preclinical AD A+/TN−" group had significantly lower CSF PGRN than the rest of the Alzheimer's *continuum* groups (Fig 3 and Table 4) but not to "healthy controls". No other group differences were found.

Given that *GRN rs5848* is a well-known modifier of PGRN levels (Rademakers *et al*, 2008; Nicholson *et al*, 2014; Morenas-Rodríguez *et al*, 2015), we repeated the same analysis but also accounting for the *rs5848* genotype, which was available for 58.5% of the cases (see Table 4). CSF PGRN still significantly differed between groups ($F_{4,342}$ = 5.66, P = 0.0002), where significant differences were observed when "AD CDR = 1" was compared to "healthy controls" (P = 0.030) and "Preclinical AD A+/TN−" (P = 0.0001) groups in Bonferroni corrected pair-wise *post hoc* comparisons. Similarly, CSF PGRN remained lower in "Preclinical AD A+/TN−" compared to "AD CDR = 0.5" (P = 0.002) and "AD CDR = 1" (P = 0.0001) groups and a tendency existed when compared to "Preclinical AD A+/TN+" (P = 0.082). Consistent with previous reports (Rademakers *et al*, 2008; Nicholson *et al*, 2014; Morenas-Rodríguez *et al*, 2015), there was a significant effect of the *GRN rs5848* genotype on CSF PGRN levels ($F_{2,342}$ = 20.6, P < 0.0001), such that the CSF PGRN mean level of the *GRN rs5848* TT carriers (M = 1348 pg/ml, SD = 330) was significantly lower than that of the *rs5848* CT carriers (M = 1524 pg/ml, SD = 277, P = 0.0001) and *rs5848* CC carriers (M = 1659 pg/ml, SD = 379, P < 0.0001) groups (Bonferroni corrected pair-wise *post hoc* comparisons). Together, the CSF PGRN changes across biomarker-defined clinical stages are consistent with the increase we found in ADAD throughout EYO.

To further confirm that CSF PGRN changes across the disease course, we also tested whether CSF PGRN levels are associated with cognitive and functional scores in those participants that fall in the Alzheimer's *continuum* category (Table 5; Fig 4). Our primary cognitive measures were the composite scores ADNI-Mem (Fig 4A), for memory performance, and ADNI-EF (Fig 4B), for executive function, since they have been previously validated in the ADNI study, are robust and have external validity (Crane *et al*, 2012; Gibbons *et al*, 2012; Habeck *et al*, 2012). We computed three linear regression models, including as main predictors: unadjusted (Model 1); adjusted for age, gender, *APOE* ε4 status and education (Model 2); and additionally adjusted for CSF $A\beta_{1-42}$ and CSF T-tau (Model 3, Table 5). Higher CSF PGRN was associated with lower memory performance as measured by ADNI-Mem (β = −0.145, P = 0.002) and executive function as assessed by ADNI-EF (β = −0.145, P = 0.002) in an unadjusted model (Model 1, Table 5 and Appendix Table S6). These associations remained significant after adjustment for age, gender, *APOE* ε4 status and education (Model 2, ADNI-Mem: β = −0.140, P = 0.002, Fig 4A; ADNI-EF: β = −0.150, P = 0.0008, Fig 4B; Table 5 and Appendix Table S6). We also studied ADAS-Cog 11, ADAS-Cog 13, MMSE and CDR-SB as secondary cognitive measures, and the results were similar (Fig 4C–F, Table 5 and Appendix Table S6). The associations remain significant after accounting for $A\beta_{1-42}$ and T-tau (Model 3) for ADNI-EF, ADAS-Cog 11 and ADAS-Cog 13, and a tendency existed for ADNI-Mem and MMSE (Table 5, Appendix Table S6), indicating that the association between CSF PGRN and cognitive scores is, at least in part, not dependent on the AD CSF core biomarkers.

We also tested whether CSF PGRN is associated with neuroimaging biomarkers of neurodegeneration typically affected in AD, namely temporo-parietal FDG-PET uptake and total hippocampal volume, in the Alzheimer's *continuum* category (Fig 5). In a linear

Table 4. ADNI participants' characteristics for the control and Alzheimer's *continuum* groups in ADNI.

		Alzheimer's *continuum* (n = 474)				
	Healthy controls (n = 128)	Preclinical AD A+/TN− (n = 56)	Preclinical AD A+/TN+ (n = 48)	AD CDR = 0.5 (n = 289)	AD CDR = 1 (n = 81)	P-value (group effect)
Age, years	72.5 (5.37)	73.2 (5.96)	76.5 (5.37)	73.3 (7.05)	73.8 (9.27)	0.017*
Female, %	48.4	46.4	52.1	42.6	50.6	0.542
APOE ε4 carriers, %	14.8	39.3	58.3	76.5	74.1	< 0.0001*
GRN rs5848 TT carriers, %[a]	5.20	13.2	6.90	13.6	14.8	0.472
Education, y	16.3 (2.79)	16.5 (2.74)	16.5 (2.47)	15.9 (2.87)	15.1 (2.78)	0.010*
Cognitive tests, scores						
ADNI-Mem	1.14 (0.59)	1.04 (0.61)	0.86 (0.56)	−0.28 (0.64)	−1.02 (0.46)	< 0.0001*
ADNI-EF	0.94 (0.75)	0.74 (0.70)	0.40 (0.61)	−0.12 (0.79)	−1.07 (0.78)	< 0.0001*
ADAS-Cog11	5.96 (3.12)	5.85 (3.06)	6.54 (3.01)	13.2 (5.34)	23.2 (6.98)	< 0.0001*
ADAS-Cog13	8.94 (4.57)	9.27 (4.51)	10.2 (4.45)	21.2 (7.38)	34.6 (7.95)	< 0.0001*
MMSE	29.1 (1.12)	28.9 (1.23)	29.0 (1.20)	26.3 (2.39)	22.7 (2.06)	< 0.0001*
CDR-SB	0.016 (0.09)	0.054 (0.16)	0.073 (0.21)	2.09 (1.09)	5.62 (1.17)	< 0.0001*
CSF biomarkers, pg/ml[b]						
T-tau	184 (31.8)	166 (41)	329 (77.5)	382 (135)	395 (137)	< 0.0001*
P-tau$_{181P}$	16.3 (2.87)	15.4 (4.09)	33.0 (8.93)	39.1 (14.5)	39.4 (15.1)	< 0.0001*
Aβ$_{1–42}$	1,455 (227)	724 (193)	712 (173)	636 (167)	575 (159)	< 0.0001*
PGRN[c]	1,502 (279)	1,394 (365)	1,569 (305)	1,541 (343)	1,649 (375)	< 0.0001*

A, amyloid-β biomarker status; Aβ$_{1–42}$, amyloid-β 42; AD, Alzheimer disease; APOE, apolipoprotein E; ADAS-Cog, Alzheimer's disease Assessment Scale—cognitive subscale; ADNI-Mem, ADNI memory composite score; ADNI-EF, ADNI executive function composite score; CDR, clinical dementia rating; CDR-SB, clinical dementia rating sum of boxes; CSF, cerebrospinal fluid; MMSE, Mini-Mental State Examination; N, neurodegeneration biomarker status; P-tau$_{181P}$, tau phosphorylated at threonine 181; T, tau pathology biomarker status; T-tau, total tau; y, years.
Data are expressed as mean (M) and standard deviation (SD) or percentage (%), as appropriate. Pearson's chi-square tests were used for the group comparisons of categorical variables and one-way ANOVA to compare continuous variables.
*Significant differences. The P-values indicated in the last column refer to the group effects in these tests.
[a]*GRN rs5848* genotype was available in 77 "healthy controls" (60%), 38 "Preclinical AD A+/TN−" (68%), 29 "Preclinical AD A+/TN+" (60%), 154 "AD CDR = 0.5" (53%) and 54 "AD CDR = 1" (67%).
[b]The CSF core biomarker measurements were performed using the electrochemiluminescence immunoassays, total-tau CSF, phospho-tau(181P) CSF and Elecsys β-amyloid(1–42) CSF. The Elecsys β-amyloid(1–42) assay has an upper technical limit of 1700 pg/ml; the values above this limit were truncated to this value.
[c]CSF PGRN levels were log-transformed and assessed by a linear model adjusted for age, gender and *APOE* ε4 status (see main text).

regression analysis adjusted for age, gender, *APOE* ε4 status and education, higher CSF PGRN was associated with lower temporo-parietal FDG-PET uptake (β = −0.133, P = 0.010) (Fig 5A), but not total hippocampal volume (β = −0.063, P = 0.151, Fig 5B).

In the second approach, we examined CSF PGRN between biomarker profile [as defined by the A/T/N classification (Jack *et al*, 2016a)] within each clinical stage (Fig EV1A). Unlike the former analysis of the Alzheimer's *continuum*, this is an unbiased comparison that does not assume any particular sequence of biomarkers in the course of the disease. Appendix Table S4 displays a summary of the demographics of the entire ADNI sample, analysed here. As expected, the CDR = 1 clinical stage had some biomarker profiles with low number of subjects that preclude any comparisons but are shown in Fig EV1A for the sake of completeness. Following this approach, we found that the "A−TN+" biomarker profiles (i.e. SNAP) had the highest CSF PGRN, which was significantly higher than the rest of the biomarker profiles within the same clinical stage (Fig EV1A). On the contrary, the "A+TN−" biomarker profiles had the lowest CSF PGRN. We next grouped the subjects that fall into each of the biomarker category of "suspected non-Alzheimer's pathophysiology" (SNAP; Table 3, highlighted in dark grey) (Jack *et al*, 2012, 2016b; Caroli *et al*, 2015; Dani *et al*, 2017) and we compared them to the healthy controls and the Alzheimer's *continuum* category as a whole. CSF PGRN significantly differ between categories ($F_{2,778}$ = 24.7, P < 0.0001, Fig EV1B), and Bonferroni corrected pair-wise *post hoc* tests showed that CSF PGRN was significantly higher in the SNAP category compared to the healthy controls (P < 0.0001) and Alzheimer's *continuum* categories (P < 0.0001). Finally, we also tested the association between CSF PGRN and cognitive function and neuroimaging biomarkers within the SNAP category, with the same linear regression models we applied in the analysis focused on the Alzheimer's *continuum* category. Strikingly, and in contrast with the results in the Alzheimer's *continuum*, CSF PGRN was not associated with cognitive decline in the SNAP group in any of the models tested (Appendix Table S7). In line with this finding, CSF PGRN was also not associated with temporo-parietal FDG-PET uptake in the SNAP group (β = +0.102, P = 0.203). These results suggest that, despite the fact that CSF PGRN is increased in SNAP, it selectively associates with disease severity in AD.

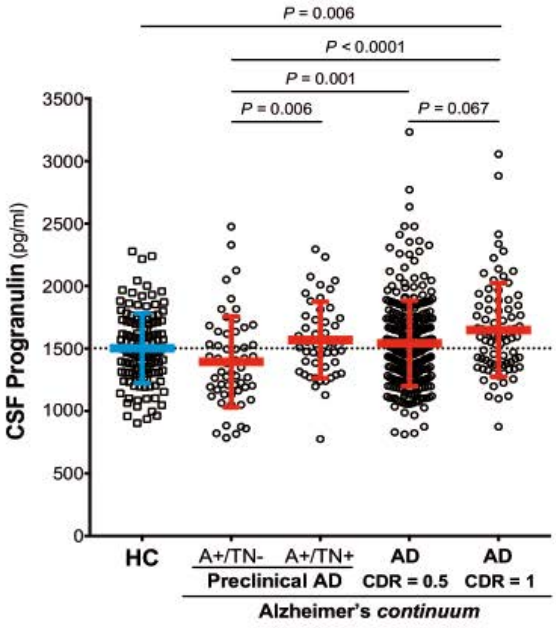

Figure 3. CSF PGRN levels across the Alzheimer's *continuum*.

Scatter plot representing the levels of CSF PGRN in healthy controls (depicted in blue) and the different stages of the Alzheimer's *continuum* (depicted in red). The blue and the red bars represent the mean and the standard deviation (SD). The analysis and graphs were performed excluding CSF PGRN outliers (1 "healthy control", 1 "Preclinical AD A+/TN−", 4 "AD CDR = 0.5" and 1 "AD CDR = 1"). Including them yielded a similar result, and CSF PGRN was still significantly higher in the "AD CDR = 1" group compared to the "healthy controls" ($P = 0.001$) and "Preclinical AD A+/TN−" ($P = 0.0001$) groups. *P*-values were assessed by a one-way analysis of covariance adjusted for age, gender and *APOE* ε4, followed by Bonferroni corrected pair-wise *post hoc* comparisons. A: amyloid-β biomarker status; AD: Alzheimer's disease; CDR: clinical dementia rating; CSF, cerebrospinal fluid; N, neurodegeneration biomarker status; T: tau pathology biomarker status.

CSF PGRN is not a clinical diagnostic biomarker for AD

We assessed the diagnostic accuracy of CSF PGRN to discriminate between AD and controls. To this regard, a receiver operating characteristic (ROC) curve analysis was undertaken (Fig EV2). The area under the curve (AUC) was 0.655 (95% CI 0.581–0.729, $P = 0.0001$, Fig EV2A) for discriminating ADAD mutation carriers from non-carriers of the DIAN study, and 0.607 (95% CI 0.528–0.686, $P = 0.009$, Fig EV2B) for discriminating late-onset AD CDR = 1 from healthy controls. Although the significant *P*-values denote that the AUC of CSF PGRN is significantly different from the area under the diagonal, which corresponds to a random performance of a test, these are low AUC that indicate a poor accuracy to discriminate between AD and controls. Together with the fact that CSF PGRN considerably overlaps between groups, these results show that, consistent with previous data (Nicholson *et al*, 2014; Körtvélyessy *et al*, 2015; Morenas-Rodríguez *et al*, 2015; Wilke *et al*, 2017), CSF PGRN is not useful as a diagnostic marker in AD.

CSF PGRN is associated with CSF sTREM2 and markers of neurodegeneration in both autosomal dominant and late-onset Alzheimer's disease

We and others previously described an increase in the microglial-derived protein sTREM2 in the CSF of both ADAD and late-onset AD patients (Heslegrave *et al*, 2016; Piccio *et al*, 2016; Suárez-Calvet *et al*, 2016a,b). Since CSF PGRN also gradually increases during the course of the disease and it is mainly produced by activated microglia (Daniel *et al*, 2000; Naphade *et al*, 2010; Petkau *et al*, 2010; Philips *et al*, 2010; Kleinberger *et al*, 2013; Suh *et al*, 2014), we investigated whether these two proteins are associated (Figs 6A and B, and 7A–C). In a linear regression model, we found that CSF PGRN and CSF sTREM2 were significantly associated in ADAD mutation carriers of the DIAN study ($\beta = +0.514$, $P < 0.0001$, Fig 6B). Similarly, CSF PGRN and sTREM2 were also significantly associated in individuals of the Alzheimer's *continuum* ($\beta = +0.344$, $P < 0.0001$, Fig 7B) and the SNAP category ($\beta = +0.296$, $P < 0.0001$, Fig 7C) of the ADNI study. Importantly, these associations only occurred in AD and SNAP since no association was found in the NC of the DIAN study ($\beta = +0.094$, $P = 0.424$, Fig 6A) and in the healthy controls of the ADNI study ($\beta = +0.106$, $P = 0.246$, Fig 7A). Together, these results suggest that, whenever there is neurodegeneration (triggered by amyloidosis or other causes), there is a concurrent release of the microglial proteins PGRN and sTREM2 into the CSF.

We also tested the associations between CSF PGRN and each of the core CSF biomarkers of AD (T-tau, P-tau$_{181P}$ and Aβ_{1-42}) in linear regression models for both ADAD (DIAN) and late-onset AD (ADNI) (Figs 6C–H and 7D–L; Appendix Table S8 summarizes the results including biomarker outliers). The results paralleled those we previously found for CSF sTREM2 (Suárez-Calvet *et al*, 2016a, b). CSF PGRN was associated with CSF T-tau and CSF P-tau$_{181P}$, markers of neurodegeneration and neurofibrillary tangle degeneration, respectively, in both ADAD mutations carriers (T-tau: $\beta = +0.258$ $P = 0.008$; P-tau$_{181P}$: $\beta = +0.191$, $P = 0.041$; Fig 6D and F) and NC (T-tau: $\beta = +0.240$ $P = 0.032$; P-tau$_{181P}$: $\beta = +0.290$, $P = 0.008$, Fig 6C and E). Interestingly, this association was only present in the ADNI subjects of the Alzheimer's *continuum* (T-tau: $\beta = +0.295$ $P < 0.0001$; P-tau$_{181P}$: $\beta = +0.280$, $P < 0.0001$; Fig 7E and H), but not in healthy controls (T-tau: $\beta = +0.157$ $P = 0.087$; P-tau$_{181P}$: $\beta = +0.118$, $P = 0.195$; Fig 7D and G) or SNAP subjects (T-tau: $\beta = +0.043$ $P = 0.564$; P-tau$_{181P}$: $\beta = +0.104$, $P = 0.172$; Fig 7F and I). These findings are consistent with the fact that CSF PGRN is only associated with cognitive impairment in the Alzheimer's *continuum* category but not in SNAP and support the idea that CSF PGRN parallels the burden of the disease specifically in AD.

In contrast, we did not observe an association between CSF PGRN and Aβ_{1-42} in either NC or MC of the DIAN sample (Fig 6G and H) and only a weak association was found in the Alzheimer's *continuum* and SNAP participants of the ADNI sample (Fig 7J–L).

Discussion

In this cross-sectional study, we found that CSF PGRN increases throughout the course of AD, both in ADAD and in late-onset AD. Furthermore, CSF PGRN is associated with the microglial-derived

Table 5. Associations of CSF PGRN with cognitive measures in the Alzheimer's *continuum* category (ADNI sample).

	Model 1 (unadjusted)		Model 2 (adjusted for age, gender, *APOE* ε4 and education)		Model 3 (also adjusted for $A\beta_{1-42}$ and T-tau)	
	β	*P*	β	*P*	β	*P*
ADNI-Mem	−0.145	0.002*	−0.140	0.002*	−0.080	0.064
ADNI-EF	−0.145	0.002*	−0.150	0.0008*	−0.116	0.001*
ADAS-Cog 11	+0.138	0.003*	+0.135	0.003*	+0.095	0.036*
ADAS-Cog 13	+0.149	0.001*	+0.148	0.001*	+0.108	0.016*
MMSE	−0.126	0.006*	−0.126	0.006*	−0.087	0.052
CDR-SB	+0.174	0.011*	+0.125	0.007*	+0.070	0.119

$A\beta_{1-42}$, amyloid-β 42; ADAS-Cog, Alzheimer's disease Assessment Scale—cognitive subscale; ADNI-Mem, ADNI memory composite score; ADNI-EF, ADNI executive function composite score; CDR-SB, clinical dementia rating sum of boxes; MMSE, Mini-Mental State Examination; T-tau, total tau.
Associations between CSF PGRN and cognitive measures were studied only in the participants of the Alzheimer's *continuum* ($n = 474$) and were assessed by three different linear regression models. The standardized regression coefficients (β) and the *P*-values are shown.
Note that higher levels of CSF PGRN are associated with worse cognitive performance in all tests investigated even when age, gender *APOE* ε4 status and education (Model 2) are accounted. The associations still remain after adding $A\beta_{1-42}$ and T-tau as added in the model (Model 3) in all tests except in ADNI-Mem, MMSE and CDR-SB.
The analysis was performed excluding CSF PGRN outliers. The same analysis including these outliers yielded similar results (Appendix Table S6). There were two subjects without ADAS-Cog11 score and six subjects without ADAS-Cog13 scores.
*Significant differences.

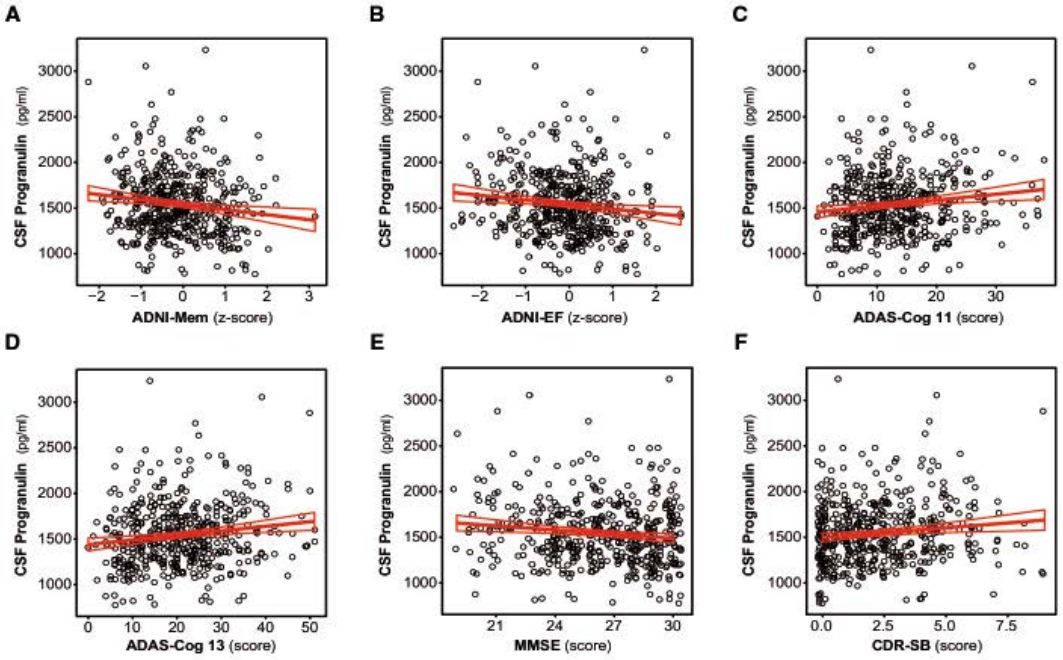

Figure 4. CSF PGRN as a function of cognitive function.

Scatter plots representing the association of CSF PGRN with different cognitive tests. Only the subjects of the Alzheimer's *continuum* group ($n = 474$) were included. In all tests studied, higher levels of CSF PGRN were associated with worse cognitive performance (namely lower scores in ADNI-Mem, ADNI-EF and MMSE and higher scores in ADAS-Cog11, ADAS-Cog13 and CDR-SB). The analysis and the graphs are excluding PGRN outliers; including them rendered similar results (Appendix Table S6). Each point depicts the value of CSF PGRN and the corresponding cognitive test score of a participant. The solid lines indicate the regression line and the 95% confidence interval (CI) calculated by a linear model (Model 1, unadjusted). Table 5 shows the standardized regression coefficients (β) and the *P*-values calculated by different models. ADAS-Cog, Alzheimer's disease Assessment Scale—cognitive subscale; ADNI-Mem: ADNI memory composite score; ADNI-EF: ADNI executive function composite score; CDR-SB: clinical dementia rating sum of boxes; MMSE, Mini-Mental State Examination.

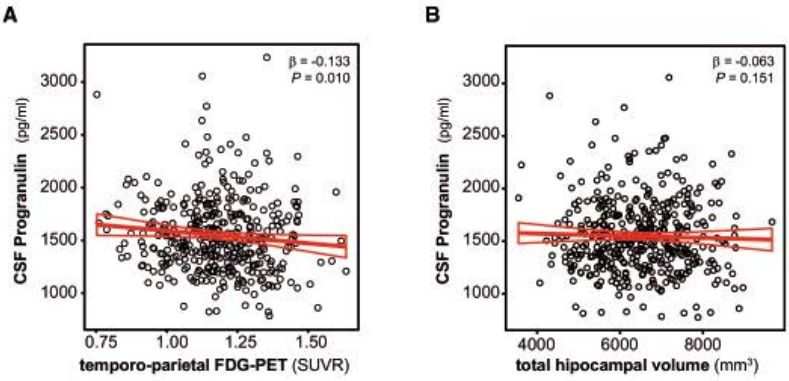

Figure 5. CSF PGRN as a function of neuroimaging biomarkers.

A, B Scatter plots representing the association of CSF PGRN with temporo-parietal FDG-PET uptake (A) and total hippocampal volume (B) within the subjects of the Alzheimer's *continuum* group (n = 474). Each point depicts the value of CSF PGRN and the corresponding neuroimaging biomarker of a participant. The solid lines indicate the regression line and the 95% confidence interval (CI). The regression coefficients (β) and the P-values calculated by a linear model adjusted for age, gender, *APOE* ε4 and education. FDG-PET: fludeoxyglucose positron emission tomography; SUVR: standardized uptake value ratio.

protein sTREM2 and with markers of neuronal injury (T-tau) and neurofibrillary tangle degeneration (P-tau$_{181P}$). Given that both PGRN and sTREM2 are predominantly expressed in microglia within the brain, our findings provide further evidence for a crucial role of microglia in modulation of onset and progression of AD. While CSF PGRN cannot serve as a diagnostic marker in AD, PGRN may serve as microglial activity marker that, together with sTREM2, could allow tracking microglial phenotypes not only during the course of the disease but also during therapeutic interventions. Furthermore, since loss of TREM2 and PGRN results in opposite phenotypes (Götzl *et al*, submitted), our findings may also allow to track different activation stages of microglia upon TREM2 or PGRN loss of function. Our study also further reinforces that PGRN is relevant not only for FTD but also for AD.

Although the results in ADAD and late-onset AD consistently show a continuous increase in CSF PGRN while disease progresses, differences between ADAD and late-onset AD were observed. While in ADAD CSF PGRN increased early in the disease (10 years before the expected symptom onset), in late-onset AD, the CSF PGRN levels did not reach a significant increase compared to healthy controls until the mild dementia stage (CDR = 1). Importantly, however, all late-onset AD groups in the Alzheimer's *continuum* had increased CSF PGRN levels compared with the earliest stage of AD, the "Preclinical AD A+TN−" group, which had the lowest CSF PGRN levels. This means that once neurodegeneration and neurofibrillary tangle degeneration have started (as expressed by the N and T positivity, respectively), CSF PGRN increases. This parallels what occurs in ADAD, where CSF T-tau (and hence, probably neurodegeneration as well) significantly increases 15 years before the symptom onset (Bateman *et al*, 2012; Fagan *et al*, 2014; Suárez-Calvet *et al*, 2016a, b) and this is followed by the later increase in CSF PGRN (EYO = −10, as shown in the present study) and CSF sTREM2 (EYO = −5; Suárez-Calvet *et al*, 2016a,b). Our conclusion is further supported by the fact that CSF PGRN is associated with CSF T-tau, P-tau$_{181P}$, cognitive impairment and temporo-parietal FDG specifically in late-onset AD, but not in SNAP. This may indicate that, although CSF PGRN production may be increased whenever there is neuronal injury, CSF PGRN is specifically coupled to the progression of neurodegeneration in AD. In contrast, CSF PGRN was not associated with hippocampal volume, which may suggest a more complex link between inflammatory-related biomarkers such as CSF PGRN and structural imaging. In fact, CSF sTREM2 was found to be positively associated with grey matter volume in mild cognitive impairment due to AD, despite the fact that CSF sTREM2 increases throughout the early stages of the disease, which was attributed to a possible brain swelling (Gispert *et al*, 2016). CSF PGRN was not either associated with cognitive measurements in ADAD, despite its increase throughout EYO and its clear association with CSF T-tau and P-tau$_{181P}$. This may suggest that in ADAD, CSF PGRN increases early in the disease but remains stable throughout

Figure 6. Association of CSF PGRN with AD core CSF biomarkers in ADAD (DIAN).

A–H Scatter plots representing the associations of CSF PGRN with CSF sTREM2 and each of the AD CSF core biomarkers (T-tau, P-tau$_{181P}$ and Aβ_{1-42}) in non-carriers (NC, blue; A, C, E and G) and in mutation carriers (MC, red; B, D, F and H). Each point depicts the value of CSF PGRN and the corresponding biomarker of a subject and the solid lines indicate the regression line and the 95% confidence interval (CI) for each of the groups. The standardized regression coefficients (β) and the P-values are shown and were computed using a linear model adjusting for age, gender and *APOE* ε4. The sample contained some outliers (defined as 3 SDs below or above the group mean) of the CSF core markers of AD. The results shown in the figure are excluding these outliers. We also performed the analysis including these outliers which yielded similar results (Appendix Table S8). Aβ_{1-42}: amyloid-β 42; T-tau: total tau; P-tau: tau phosphorylated at threonine 181.

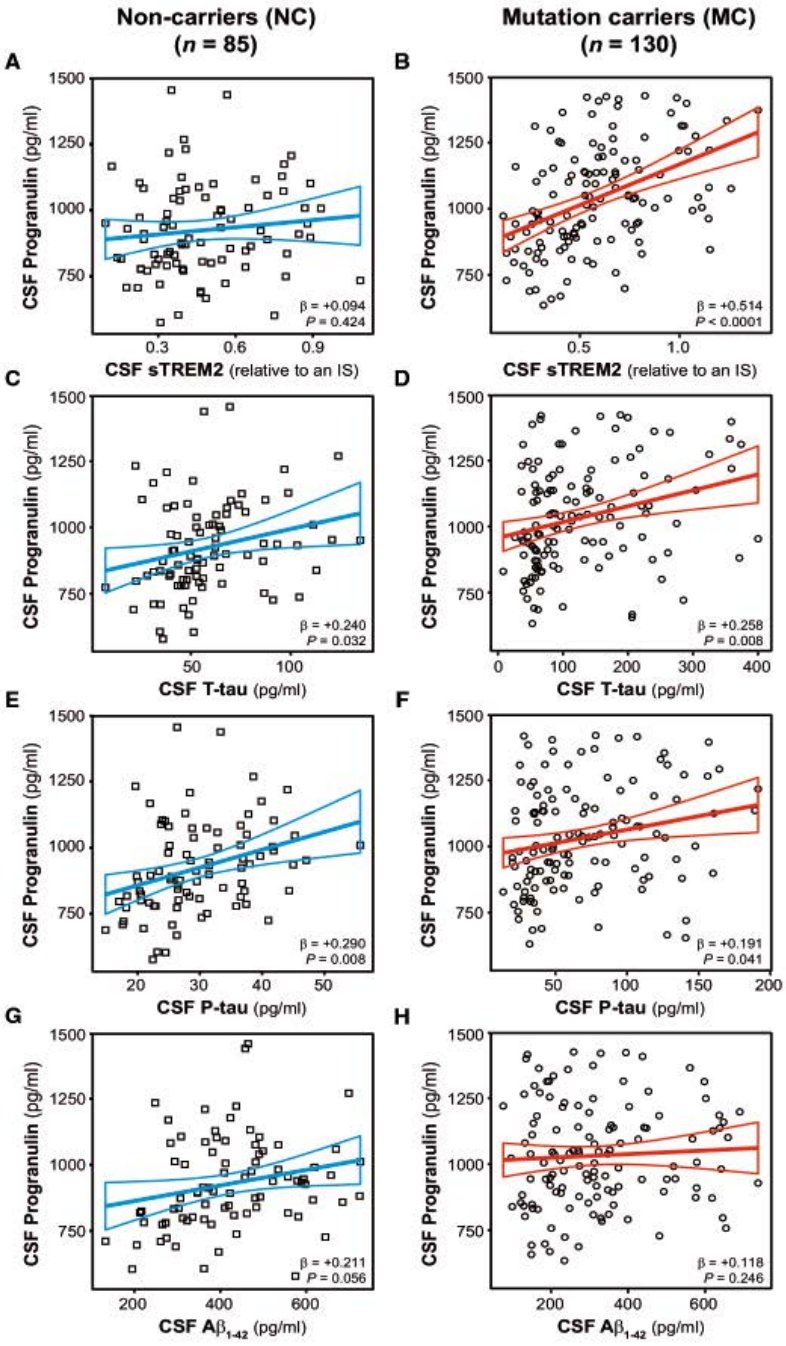

Figure 6.

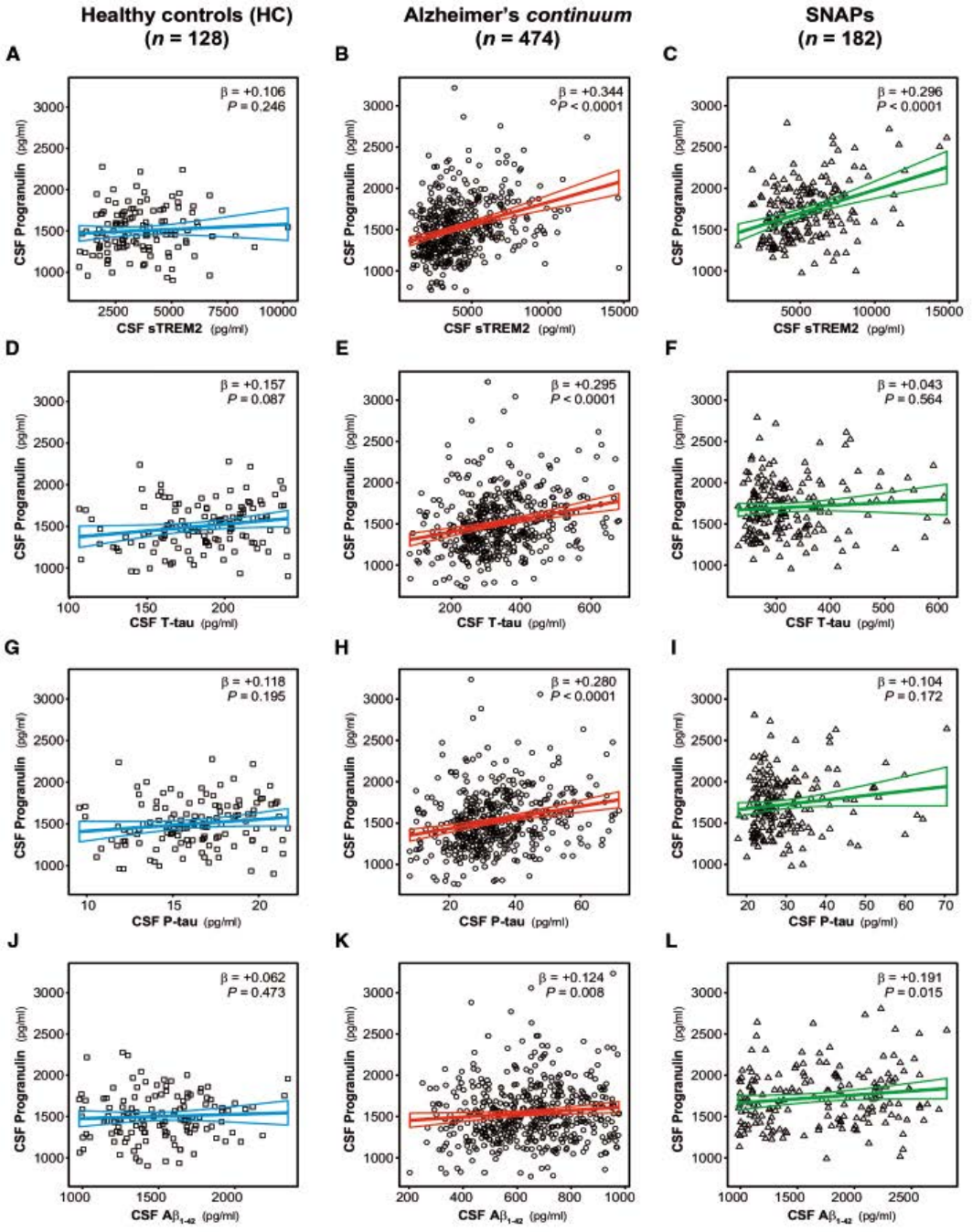

Figure 7.

Figure 7. Association of CSF PGRN with AD core CSF biomarkers in late-onset AD (ADNI).

A–L Scatter plots representing the associations of CSF PGRN with CSF sTREM2 and each of the AD CSF core biomarkers (T-tau, P-tau$_{181P}$ and Aβ_{1-42}) in healthy controls (blue; A, D, G and J), Alzheimer's *continuum* (red; B, E, H and K) and "suspected non-Alzheimer's pathophysiology (SNAP)" groups (green; C, F, I and L). Each point depicts the value of CSF PGRN and the corresponding biomarker of a subject, and the solid lines indicate the regression line and the 95% confidence interval (CI) for each of the groups. The standardized regression coefficients (β) and the *P*-values are shown and were computed using a linear model adjusting for age, gender and *APOE* ε4. The sample contained some outliers (defined as 3 SDs below or above the group mean) of the CSF core markers of AD, and the analysis including these outliers yielded similar results (Appendix Table S8). The Aβ_{1-42} values used for the association test are those based on an extrapolation curve since the upper technical limit is 1,700 pg/ml. We also tested the associations with Aβ_{1-42} values truncated at the upper technical limit and the result was similar. Aβ_{1-42}: amyloid-β 42; T-tau: total tau; P-tau: tau phosphorylated at threonine 181; SNAP: suspected non-Alzheimer's pathophysiology.

the later clinical progression. In ADAD, the causative *PSEN1*, *PSEN2* and *APP* mutations are such strong driving forces of disease pathology that other factors, such as PGRN, may be less associated with cognition.

In contrast to our findings, previous studies did not detect an increase of CSF PGRN in AD (Nicholson *et al*, 2014; Körtvélyessy *et al*, 2015; Morenas-Rodríguez *et al*, 2015; Wilke *et al*, 2017). This is likely due to the fact that DIAN and ADNI provide a number of samples and an exhaustive clinical data that is unrivalled by any other cohort. In DIAN, we can compare mutation carriers, which are destined to develop AD, with the best controls possible, that is, their non-carriers' siblings. A further advantage of studying ADAD is the use of the concept of estimated years from expected symptom onset (EYO) as a proxy of disease evolution to predict a temporal progression of CSF PGRN, despite the fact that this is a cross-sectional study. This approach has been well validated by studies of the DIAN consortium that have shown that there is a high correlation between the parental age of onset and the mean age at onset of the affected family members (from which EYO is derived) and the actual age of onset (Ryman *et al*, 2014). Finally, in our study we applied an unbiased approach to classify the participants of ADNI, based on biomarker profile (A/T/N) and cognition (C), which made us independent of different diagnostic schemes in different centres (Jack *et al*, 2016a). That allowed us to study those groups that are included in the Alzheimer's *continuum*, from preclinical to mild dementia stages of the disease, and compare them with the evolution of the disease in ADAD as defined by EYO.

The precise mechanism underlying the increase in CSF PGRN during AD has yet to be determined. Since PGRN is highly increased upon activation of microglia (Petkau *et al*, 2010), we speculate that increased CSF PGRN reflects elevated microglial function. It is well established that activation of microglia occurs in AD (Lyman *et al*, 2014; Heneka *et al*, 2015) and, in fact, we and others have previously shown that CSF sTREM2 also increases throughout the evolution of the disease (Heslegrave *et al*, 2016; Piccio *et al*, 2016; Suárez-Calvet *et al*, 2016a,b). A remarkable finding of this study is that higher CSF PGRN levels are associated with higher CSF sTREM2 levels exclusively when disease occurs (either in ADAD MC, late-onset AD or in SNAP) but not in controls. This may indicate that these two proteins are released by microglia adopting a disease-associated signature (Keren-Shaul *et al*, 2017; Krasemann *et al*, 2017). However, it is also possible that these two proteins reflect different populations of microglia co-existing in the disease and may even have opposing effects on disease. While lack of TREM2 locks microglia in a homeostatic state (Mazaheri *et al*, 2017), lack of PGRN locks microglia in a hyperactive state (Götzl *et al*, submitted). Whether these responses are beneficial or detrimental in the disease may depend on the stage of the disease and needs to be addressed in longitudinal studies. The fact that CSF PGRN is associated with the microglial-derived protein CSF sTREM2 and both are associated with markers of neuronal injury and neurofibrillary tangle degeneration suggests that elevated levels of CSF PGRN and CSF sTREM2 early during the disease reflect a microglial response to neuronal injury. In line with this, recent studies demonstrated that disease-associated *TREM2* variants cause a loss of function suggesting that TREM2 is protective (Kleinberger *et al*, 2014, 2017; Wang *et al*, 2015; Song *et al*, 2018). Similarly, haploinsufficiency of PGRN causes FTD (Baker *et al*, 2006; Cruts *et al*, 2006), and a complete loss of PGRN results in a lysosomal storage disorder with severe neurodegeneration (Smith *et al*, 2012; Götzl *et al*, 2014). Therefore, TREM2 and PGRN may have a protective function at least early during the disease. TREM2 clearly has a cell-autonomous function mediated via DAP12 signalling within microglia (Colonna & Wang, 2016). However, sTREM2, which is released on the plasma membrane, may also have non-cell-autonomous functions. Similarly, PGRN is either targeted directly from the trans-Golgi to lysosomes (where it then may have cell-autonomous functions) or released via the secretory pathway (Götzl *et al*, 2016). In that case PGRN, like sTREM2, may have intrinsic functions within microglia, but both proteins may be able to affect cellular functions in non-microglial cells as well.

Finally, it is important to note that CSF PGRN has no utility as a diagnostic marker in AD since the values overlap considerably between groups. This study has to be interpreted from the perspective of the pathophysiological importance of PGRN in AD, and not as the evaluation of a putative diagnostic biomarker. Taken together, PGRN and sTREM2 may both be increased early in the disease upon an initial response of microglia to first neuronal injury. Their CSF levels may therefore allow conclusions about the functional status of microglia. This is also of specific value for the investigation of novel strategies aiming to modulate microglial activity.

Materials and Methods

DIAN participants and study design

The DIAN study is a multicentre study initiated in 2008 that aims to develop a registry of families with known ADAD mutations (namely *PSEN1*, *PSEN2* or *APP*) and investigate the pathophysiological changes that occur in the different stages of AD (Bateman *et al*, 2012; Morris *et al*, 2012; Suárez-Calvet *et al*, 2016a,b). We measured CSF PGRN from a total of 218 participants, 131 mutation carriers (MC) and 87 non-carriers (NC). All DIAN participants underwent a comprehensive clinical and neuropsychological evaluation (Bateman *et al*, 2012; Storandt *et al*, 2014). CSF collection

follows standard procedures (Fagan *et al*, 2014; Monserrate *et al*, 2015), and the measurements of T-tau, P-tau$_{181P}$ and CSF Aβ_{1-42} were performed by the Luminex bead-based multiplexed xMAP technology (INNO-BIA AlzBio3, Innogenetics; Fagan *et al*, 2014). The estimated years from expected symptom onset (EYO) is calculated as the difference between the participant's age at evaluation and the mean age at onset of all other affected family members (Bateman *et al*, 2012; Ryman *et al*, 2014).

ADNI participants and study design

The ADNI project (http://adni.loni.usc.edu) is a multicentre longitudinal study led by Principal Investigator Michael Weiner and with the main goal to develop and validate biomarkers for subject selection and as surrogate outcome measures in late-onset AD (Weiner *et al*, 2017). Inclusion and exclusion criteria for the ADNI study were previously described comprehensively (Petersen *et al*, 2010).

For the present study, we measured CSF PGRN in a cross-sectional sample of the ADNI project consisting of a total of 1,028 individuals. The criteria to select the participants are described in the Appendix Table S9. The CSF PGRN measurements were uploaded to the ADNI database (http://adni.loni.usc.edu) on 16/03/2018, and all the data from ADNI used in this study were downloaded on 21/03/2018.

The CSF core biomarker measurements in ADNI were performed using the electrochemiluminescence immunoassays Elecsys total-tau CSF, phosphor-tau(181P) CSF and β-amyloid (1–42) CSF on a fully automated Elecsys cobas e 601 instrument (Roche) and a single lot of reagents for each of the three measured biomarkers (provided in UPENNBIOMK9.csv file available at http://adni.loni.usc.edu). These immunoassays are for investigational use only. They are currently under development by Roche Diagnostics and not commercially available yet. The Elecsys β-amyloid (1–42) CSF assay measuring range beyond the upper technical limit has not been formally established. Therefore, use of values above the upper technical limit, which are provided based on an extrapolation of the calibration curve, is restricted to exploratory research purposes and is excluded for clinical decision making or for the derivation of medical decision points. The analyte measuring ranges (lower technical limit to upper technical limit) of these assays are the following: 80–1,300 pg/ml for total-tau CSF, 8–120 pg/ml for phosphor-tau(181P) and 200–1,700 pg/ml for Elecsys β-amyloid (1–42) CSF immunoassays. There were 160 values of Aβ_{1-42} that were above the upper technical limit and were truncated to 1,700 pg/ml. There were no samples with T-tau or P-tau$_{181P}$ values above its respective upper technical limit of quantification. For Aβ_{1-42}, exploratory measurements are available based on the extrapolation of the calibration curve. The associations of the CSF biomarkers described in the text are using these extrapolated values. The analysis using the Aβ_{1-42} truncated values and those using the extrapolated measurements yielded similar results.

We classified the ADNI participants in a descriptive unbiased approach (Jack *et al*, 2016a) into two different schemes. First, their biomarker profile, which is defined by the three different pathologic processes that occur in AD and that a biomarker can measure, that is: (i) aggregated Aβ (A, as defined by CSF Aβ_{1-42}); (ii) aggregated tau (T, as defined by CSF P-tau$_{181P}$); (iii) neurodegeneration or neuronal injury (N, as defined by CSF T-tau). We binarized each of the biomarker group into positive (+, abnormal) or negative (−, normal) based on the reported cut-offs for each of the biomarkers (Hansson *et al*, 2018). Levels below 976.6 pg/ml (Aβ_{1-42}) or above 21.8 pg/ml (P-tau$_{181P}$) and 245 pg/ml (T-tau) were categorized as positive (A+, T+, N+, respectively). To reduce the number of groups, we merged the aggregated tau (T) and neurodegeneration (N) groups. If either aggregated tau (T) or neurodegeneration (N) was abnormal (T+ or N+), participants were classified as TN abnormal (TN+). If both aggregated tau (T) and neurodegeneration (N) were normal (T− and N−), participants were classified as TN normal (TN−). Note that in the "healthy control" group, we set the criteria that both aggregated tau (T) and neurodegeneration (N) biomarker profile should be normal, so that we ensure that this group is indeed free of pathology. Importantly, only 54 participants (5.3% of the total sample) displayed discrepancies between the T and N biomarker groups. Second, we classified the participants based on clinical symptoms based on the clinical dementia rating (CDR) global score (Morris, 1993) into cognitively unimpaired (CDR = 0), very mild dementia (CDR = 0.5) and mild dementia (CDR = 1). In the sample studied, there were no participants with moderate or severe dementia (CDR = 2–3). The combination of both the biomarker and the clinical classification rendered 12 different groups that are summarized in Table 3.

Cognition in ADNI participants was assessed by ADNI-Mem, ADNI-EF, ADAS-Cog 11, ADAS-Cog 13, MMSE and CDR-SB. ADNI-Mem and ADNI-EF are both composite scores developed by ADNI. ADNI-Mem is derived from of the following test scores: Rey Auditory Verbal Learning test, Alzheimer's Disease Assessment Schedule-Cognition (ADAS-Cog), MMSE and Logical Memory (Crane *et al*, 2012). The ADNI-EF is derived from the following tests: Wechsler Adult Intelligence Scale—Revised Digit Symbol Substitution, Digit Span Backwards, Trials A and B, Category Fluency and Clock Drawing (Gibbons *et al*, 2012).

Hippocampal volumes based on FreeSurfer segmentation and FDG-PET ROI SUVRs derived from meta-analytically regions of AD-associated hypometabolism located within the angular gyrus, posterior cingulate and inferior temporal lobe were downloaded from the ADNI database. The MRI and FDG-PET ROI segmentation has been described in detail previously (Fischl *et al*, 2002; Landau *et al*, 2011). Hippocampal volumes were further adjusted for intracranial volume using linear regression, following previous recommendations (Jack *et al*, 2017).

Ethical considerations

The study was approved by the institutional review board (IRB) of all participating centres in DIAN and ADNI, as well as our local IRB (LMU). All study participants (or their relatives) provided written informed consent.

CSF progranulin (PGRN) measurement

CSF PGRN was measured by an ELISA protocol previously established by our group using the MSD Platform (Capell *et al*, 2011). The ELISA consists of a Streptavidin-coated 96-well plates (MSD Streptavidin Gold Plates, cat. no. L15SA); a biotinylated polyclonal goat anti-human PGRN capture antibody (BAF2420, R&D Systems; 0.2 μg/ml, 25 μl/well); a monoclonal mouse anti-human PGRN

detection antibody (MAB2420, R&D Systems; 0.25 μg/ml, 25 μl/well); and a SULFO-TAG-labelled goat polyclonal anti-mouse IgG secondary antibody (MSD, cat. no. R32AC; 0.5 μg/ml, 25 μl/well). All antibodies were diluted in 0.5% bovine serum albumin (BSA) and 0.05% Tween-20 in PBS buffer (pH = 7.4). Recombinant human PGRN protein (His Tag PGRN—Sino Biological, cat. no. 10826-H08H) was used as a standard (15.6–2,000 pg/ml). In brief, Streptavidin-coated 96-well plates were blocked overnight at 4°C in blocking buffer [0.5% BSA and 0.05% Tween-20 in PBS (pH = 7.4)]. The plates were next incubated with the capture antibody for 1 h at room temperature (RT). They were subsequently washed four times with washing buffer (0.05% Tween-20 in PBS) and incubated for 2 h at RT with the CSF and the internal standard (IS) samples diluted 1:2.5 in assay buffer [0.25% BSA and 0.05% Tween-20 in PBS (pH = 7.4)] or the recombinant human PGRN protein for the standard curve also diluted in assay buffer. CSF samples were randomly distributed across plates and measured in triplicates (DIAN samples) or in duplicates (ADNI samples). The operators were blinded to the clinical information. Plates were again washed four times with washing buffer before incubation for 1 h at RT with the detector antibody. After four additional washing steps, plates were incubated with the secondary antibody for 1 h in the dark. Last, plates were washed four times with washing buffer followed by two washing steps in PBS. The electrochemical signal was developed by adding MSD read buffer T (cat. no. R-92TC) and the light emission measured using the MESO QuickPlex SQ 120. The ELISA showed good accurate results in the spike recovery (98%) experiments and minimal measurement variation (CV = 4%) between freeze–thaw cycles (nine cycles).

The measurement of all the DIAN samples was performed in a single day (22/11/2016), and three CSF samples (internal standards, IS) were loaded in all plates. All IS used in this study consisted of pooled CSFs from diagnostic clinical routine leftovers from the Ludwig-Maximilians-Universität München (LMU) Department of Neurology (Munich, Germany). All patients gave their written consent, and the study was approved by the local IRB. The interplate coefficient of variation (CV) for each of the IS was 3.6, 5.4 and 6.7%. The mean intraplate CV was 2.1%, and all replicate measures had a CV ≤ 15%. The raw values are provided as pg/ml.

The measurements of the ADNI samples were performed on four different days (between the 27/11/2017 and 06/12/2017). Four CSF-IS samples were loaded in all plates. The interplate CV for each of the IS was 4.3, 3.8, 3.4 and 5.4%. The mean intraplate CV was 2.2%, and all replicate measures had a CV ≤ 15%. Given that the ADNI measurements were done in several days, we corrected the raw measurements based on values of the four IS that were loaded on all plates. The concentration of each IS in an individual plate (plate x) was expressed as a percentage of the mean concentration across all plates as follows:

a1 (%) in plate x = [(concentration of IS1 in plate x)/(mean concentration of IS1 in all plates)] × 100
a2 (%) in plate x = [(concentration of IS2 in plate x)/(mean concentration of IS2 in all plates)] × 100
a3 (%) in plate x = [(concentration of IS3 in plate x)/(mean concentration of IS3 in all plates)] × 100
a4 (%) in plate x = [(concentration of IS4 in plate x)/(mean concentration of IS4 in all plates)] × 100

The mean of the percentages (Ax) for all the IS (a1, a2, a3, a4) in plate x was calculated, and the following correction factor was computed for each individual plate:

Correction factor for plate x = 100/Ax

The raw values were multiplied by the correction factor of the corresponding plate; the corrected values are provided as the variable "MSD_PGRNCORRECTED" in the ADNI database. Due to the low interplate CV, the corrected and raw PGRN values in the ADNI sample are highly correlated (Spearman ρ = 0.985; $P < 0.0001$).

The mean CSF PGRN level of our MSD-based assay (1,007 pg/ml) was similar to that published with other assays (Huchtemann *et al*, 2015; Zhou *et al*, 2015a; Berghoff *et al*, 2016; Meeter *et al*, 2016; Kimura *et al*, 2017; Schreiber *et al*, 2018), albeit lower than those measured by the more widely used commercial assay from Adipogen (Ghidoni *et al*, 2008; De Riz *et al*, 2010; Vercellino *et al*, 2011; Nicholson *et al*, 2014; Morenas-Rodríguez *et al*, 2015; Feneberg *et al*, 2016; Molgaard *et al*, 2016; Willemse *et al*, 2016; Wilke *et al*, 2017). We therefore compared our MSD-based ELISA with that from Adipogen (cat. no. AG-45A-0018YEK-KI01, Seoul, Korea). We prepared 39 different CSF pool samples from leftovers of the LMU Department of Neurology, as described above. We measured these CSF samples simultaneously in both the MSD-based and the Adipogen assays, following the manufacturer's instructors, and each sample in duplicate. The standard curves for each of the assays are shown in Appendix Fig S1A and B. As expected, the mean levels of CSF PGRN were lower in the MSD-based assay (mean = 1430 pg/ml, SD = 220) compared to the Adipogen assay (mean = 4,400 pg/ml, SD = 900), but they were highly correlated between them (Spearman ρ = 0.74; $P < 0.0001$; Appendix Fig S1C).

CSF sTREM2 measurement

CSF sTREM2 measurements from the DIAN study were previously reported (Suárez-Calvet *et al*, 2016a,b) and are expressed relative to an IS. Measurements of the ADNI samples were done with the same ELISA protocol with minor changes. Briefly, the assay is based on the MSD platform and it is comprehensively described in previous publications (Kleinberger *et al*, 2014; Suárez-Calvet *et al*, 2016a,b). The assay consists of a Streptavidin-coated 96-well plates (MSD Streptavidin Gold Plates, cat. no. L15SA); a biotinylated polyclonal goat IgG anti-human TREM2 antibody (R&D Systems, cat. no. BAF1828; 0.25 μg/ml, 25 μl/well) as capture antibody, which is raised against amino acids 19-174 of human TREM2; a monoclonal mouse IgG anti-human TREM2 antibody (Santa Cruz Biotechnology, B-3, cat. no. sc373828; 1 μg/ml, 50 μl/well) as a detection antibody, which is raised against amino acids 1–160 of human TREM2; and a SULFO-TAG-labelled goat polyclonal anti-mouse IgG secondary antibody (MSD, cat. no. R32AC; 0.5 μg/ml, 25 μl/well). All antibodies were diluted in 1% BSA and 0.05% Tween-20 in PBS buffer (pH = 7.4). Recombinant human TREM2 protein (Hölzel Diagnostika, cat. no. 11084-H08H), corresponding to the extracellular domain of human TREM2 (amino acids 19–174), was used as a standard (62.5–8,000 pg/ml). In brief, Streptavidin-coated 96-well plates were blocked overnight at 4°C in blocking buffer [3% bovine serum albumin (BSA) and 0.05% Tween-20 in PBS (pH = 7.4); 300 μl/well]. The plates were next incubated with the capture antibody for

1 h at RT. They were subsequently washed four times with washing buffer (200 µl/well; 0.05% Tween-20 in PBS). Thereafter, the recombinant human TREM2 protein (standard curve), the blanks, and the CSF and the internal standard (IS) samples (duplicates; dilution factor: 4) were diluted in assay buffer [0.25% BSA and 0.05% Tween-20 in PBS (pH = 7.4)] supplemented with protease inhibitors (Sigma; Cat. # P8340) and incubated (50 µl/well) for 2 h at RT. This dilution was previously selected because it showed the best recovery and linearity performance (Kleinberger *et al*, 2014). Plates were again washed four times with washing buffer before incubation for 1 h at RT with detection antibody. After four additional washing steps, plates were incubated with SULFO-tag conjugated secondary antibody for 1 h in the dark at RT. Last, plates were washed four times with washing buffer followed by two washing steps in PBS. The electrochemical signal was developed by adding 150 µl/well MSD read buffer T (cat. no. R-92TC) and the light emission measured using the MESO QuickPlex SQ 120. Raw values are provided as pg/ml.

All CSF samples were distributed randomly across plates, measured in duplicate and simultaneously to CSF PGRN (i.e. between 27/11/2017 and 06/12/2017). The mean intraplate CV was 3.1%, and all duplicate measures had a CV < 15%. Alike the CSF PGRN ELISA, four CSF IS samples were loaded in all plates. The interplate CV for each of the IS was 11.4, 12.2, 10.5 and 7.1%. We corrected the raw measurements based on values of the four IS that were loaded on all plates in a similar manner as in CSF PGRN measurements. Consequently, the corrected values were used and are available in the ADNI database as variables "MSD_sTREM2CORRECTED".

Statistical analysis

In both DIAN and ADNI samples, we only included in our study participants that had the following data available: age, gender and the three AD CSF core biomarkers ($A\beta_{1-42}$, T-tau, $P\text{-tau}_{181P}$). Within each of the samples, we determined the CSF PGRN outliers, as defined as values differing 3 standard deviations from the mean (3 outliers in the DIAN sample and 11 in the ADNI sample). In order to rule out that our results are not driven by extreme values, all the analysis described in the main text are performed without these outliers. Nevertheless, including them did not affect the main results (as shown in the main text and in Appendix Tables S1, S3, S5, S6 and S8). In the DIAN sample, CSF PGRN followed a normal distribution as assessed by visual inspection of histogram and after testing by Kolmogorov–Smirnov test ($P = 0.062$). In contrast, CSF PGRN in ADNI did not follow a normal distribution (Kolmogorov–Smirnov test, $P = 0.0001$). After a $\log_{10}$ transformation, it followed a normal distribution (Kolmogorov–Smirnov test, $P = 0.200$). All the statistical analyses in the ADNI sample were hence performed with the $\log_{10}$-transformed values.

The data from the DIAN sample (Data Freeze 9) were studied in a similar approach to that we previously applied (Suárez-Calvet *et al*, 2016a,b). In brief, comparisons of demographic, clinical and biochemical data between NC and MC were performed by Pearson's chi-square tests or *t*-tests, as appropriate. A linear regression analysis was used to test the effect of the mutation status, age, gender and *APOE* ε4 status in the levels of CSF PGRN (Appendix Table S1). In additional linear effects models, we compared the levels of CSF PGRN between carriers of the three mutated genes (i.e. *PSEN1*, *PSEN2* and *APP*).

In order to test how CSF PGRN levels change as a function of EYO (as shown in Table 2 and Fig 2), we used a similar approach to that previously published (Bateman *et al*, 2012; Fagan *et al*, 2014; Suárez-Calvet *et al*, 2016a,b). We constructed a linear mixed model with mutation status, EYO (and its interaction with mutation status) and gender as fixed effects and family affiliation as random effect. Next, we performed a polynomial regression analysis including EYO quadratic (EYO^2) and cubic (EYO^3) terms and their interactions with mutation status. We determined the model that best fitted the data by forward selection of the predictors, and the final model was chosen based on the Akaike information criterion (AIC; a lower AIC indicating a better fit; see Appendix Table S2). For CSF PGRN, the linear model (first-order EYO) showed the best fit. In previous studies, we used linear mixed-effects models with family membership as a random effect to adjust for differences in biomarker levels between families. Here, we found no significant differences in CSF PGRN levels between families ($F_{93,114} = 1.04$, $P = 0.426$) and we therefore used simple linear regression in all the following analysis.

We computed the estimated levels of CSF PGRN at each 5-year interval of EYO based on the established regression models, and we determined the group differences between MC and NC for each 5-year EYO interval by *t*-tests, as done in previous DIAN studies (Bateman *et al*, 2012; Fagan *et al*, 2014; Suárez-Calvet *et al*, 2016a,b). Comparisons between MC and NC were restricted at EYO ranging from −25 to +10, due to the low number of subjects at more extreme values of EYO.

In order to represent the progression of CSF PGRN throughout the evolution of the disease and compare it with other biomarkers and cognitive measures, we followed the approach of previous DIAN studies (Bateman *et al*, 2012; Suárez-Calvet *et al*, 2016a,b); that is, the predicted difference between MC and NC at each EYO generated by the same final linear mixed-effects model described above was divided by the standard deviation of clinical, cognitive, imaging and biochemical measures of the pooled sample, so that all variables were in a standardized and comparable scale (Fig 2B, Appendix Table S2). These figures were built with SAS software (SAS Institute).

We compared CSF PGRN levels between NC and MC in different clinical stages (determined by global CDR) in an analysis of covariance (ANCOVA) controlling for age, gender, *APOE* ε4 and education, followed by *post hoc* least significant difference (LSD) for pairwise comparisons. Individuals falling into CDR = 1 to CDR = 3 were grouped together due to the low number of subjects in these stages.

In ADNI, demographic, clinical and biochemical data group comparisons were performed by one-way ANOVA or Pearson's chi-square tests, as appropriate. To test whether CSF PGRN changed across the Alzheimer's *continuum*, we applied an ANCOVA including the biomarker-defined stages of AD, gender and *APOE* ε4 status as fixed effects and age as covariate, followed by Bonferroni corrected pair-wise *post hoc* comparisons. An additional analysis was performed adding the *GRN rs5848* genotype as a covariate. A similar approach was used for comparisons between biomarker categories shown in Fig EV1A.

To study the associations between CSF PGRN and cognitive and functional scores, we applied three linear regression models. Model

The paper explained

Problem
Alzheimer's disease (AD) is the most frequent neurodegenerative disorder. Besides β-amyloid and tau deposits, all AD brains invariantly show neuroinflammatory symptoms associated with microgliosis. Progranulin (PGRN), a secreted protein whose loss of function is associated with frontotemporal lobar degeneration and neuronal ceroid lipofuscinosis, is also genetically involved in AD. In the brain, PGRN is predominantly expressed in microglia. In this study, we investigated PGRN concentration in the cerebrospinal fluid (CSF) of two of the largest and best characterized AD patient cohorts, namely the Dominant Inherited Alzheimer's Disease Network (DIAN), which includes families with autosomal dominant AD (ADAD), and the Alzheimer's Disease Neuroimaging Initiative (ADNI), which studies the evolution of late-onset AD.

Results
In carriers of ADAD causing dominant mutations (DIAN), we found that CSF PGRN increased 10 years before the expected symptom onset. In late-onset AD (ADNI), higher CSF PGRN was associated with more advanced disease stages as well as cognitive impairment. CSF PGRN was associated with levels of proteolytically generated soluble TREM2 (sTREM2), a protein described to be a central regulator of microglial function and known to increase 5 years before the expected symptom onset. Importantly, the association between CSF sTREM2 and CSF PGRN was only observed when there was underlying pathology, but not in controls.

Impact
CSF PGRN together with CSF sTREM2 may serve as a microglia activity marker in AD and could be used to prove target engagement in clinical trials aiming to modulate microglial activity.

1 was unadjusted, Model 2 included age, gender, *APOE* ε4 and education as covariates, and Model 3 included the former covariates and also CSF $A\beta_{1-42}$ and CSF T-tau.

The association between CSF PGRN and temporo-parietal FDG-PET and total hippocampal volume was tested with a linear regression adjusted for age, gender, *APOE* ε4 and education. Analyses including hippocampal volume were adjusted for intracranial volume.

The diagnostic value of CSF PGRN to discriminate AD (ADAD or late-onset AD) from controls was tested with a receiver operating characteristic (ROC) analysis. We computed areas under the curve (AUC), and we tested whether they were significantly different from the null hypothesis that the AUC equals 0.50, which corresponds to a random test.

Finally, the association between CSF PGRN and CSF sTREM2 and the CSF core biomarkers for AD (T-tau, $P\text{-tau}_{181P}$, $A\beta_{1-42}$) was studied with a linear model adjusted for age, gender and *APOE* ε4 status. The analysis was performed stratifying for the mutation status (DIAN) or classifying the subjects in the healthy controls, Alzheimer's *continuum* or SNAP categories (ADNI). The standardized regression coefficients (β) are reported. In order to rule out that the associations were driven by extreme values, we performed the analysis both including and excluding biomarker outliers (defined as AD CSF core biomarkers 3 standard deviations below or above the group mean) and the analysis yielded similar results (Appendix Table S8).

Statistical analysis was performed in SPSS IBM, version 20.0, statistical software and the free statistical software R (http://www.r-project.org/). Figures were built using GraphPad Prism or R. All tests were two-tailed, with a significance level of $\alpha = 0.05$.

Expanded View for this article is available online.

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Author contributions
MS-C, AC, EM-R, KF, GK, EE, YD and LP performed the experiments. MS-C, AC, MAAC, EM-R, NF, YD, LP, CC, CMK, ME and CH analysed and interpreted the data. YD, LP, CMK and CC involved in extracting the genetic data. KP, RJB, AMF, JCM, JL, AD, MJ, CLM, MNR, JMR, LMS, JQT and MW contributed with patient samples and/or data. MS-C, ME and CH designed the study and wrote the manuscript. All authors critically reviewed and approved the final manuscript.

Conflict of interest
C.H. collaborates with DENALI Therapeutics. The remaining authors declare that they have no conflict of interest.

For more information
All data of the ADNI study (including the CSF PGRN and sTREM2 reported in this study) are publicly available in http://adni.loni.usc.edu/.

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Annex 6

Article: Early increase of CSF sTREM2 in Alzheimer's disease is associated with tau related neurodegeneration but not with amyloid-β pathology

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Early increase of CSF sTREM2 in Alzheimer's disease is associated with tau related-neurodegeneration but not with amyloid-β pathology

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Abstract

Background: TREM2 is a transmembrane receptor that is predominantly expressed by microglia in the central nervous system. Rare variants in the *TREM2* gene increase the risk for late-onset Alzheimer's disease (AD). Soluble TREM2 (sTREM2) resulting from shedding of the TREM2 ectodomain can be detected in the cerebrospinal fluid (CSF) and is a surrogate measure of TREM2-mediated microglia function. CSF sTREM2 has been previously reported to increase at different clinical stages of AD, however, alterations in relation to Amyloid β-peptide (Aβ) deposition or additional pathological processes in the amyloid cascade (such as tau pathology or neurodegeneration) remain unclear. In the current cross-sectional study, we employed the biomarker-based classification framework recently proposed by the NIA-AA consensus guidelines, in combination with clinical staging, in order to examine the CSF sTREM2 alterations at early asymptomatic and symptomatic stages of AD.

Methods: A cross-sectional study of 1027 participants of the Alzheimer's Disease Imaging Initiative (ADNI) cohort, including 43 subjects carrying *TREM2* rare genetic variants, was conducted to measure CSF sTREM2 using a previously validated enzyme-linked immunosorbent assay (ELISA). ADNI participants were classified following the A/T/N framework, which we implemented based on the CSF levels of $A\beta_{1-42}$ (A), phosphorylated tau (T) and total tau as a marker of neurodegeneration (N), at different clinical stages defined by the clinical dementia rating (CDR) score.

Results: CSF sTREM2 differed between *TREM2* variants, whereas the p.R47H variant had higher CSF sTREM2, p.L211P had lower CSF sTREM2 than non-carriers. We found that CSF sTREM2 increased in early symptomatic stages of late-onset AD but, unexpectedly, we observed decreased CSF sTREM2 levels at the earliest asymptomatic phase when only abnormal Aβ pathology (A+) but no tau pathology or neurodegeneration (TN-), is present.

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Conclusions: Aβ pathology (A) and tau pathology/neurodegeneration (TN) have differing associations with CSF sTREM2. While tau-related neurodegeneration is associated with an increase in CSF sTREM2, Aβ pathology in the absence of downstream tau-related neurodegeneration is associated with a decrease in CSF sTREM2.

Keywords: Alzheimer's disease, Biomarkers, Microglia, Neurodegeneration, Neuroinflammation, Shedding, TREM2

Background

The triggering receptor expressed on myeloid cells 2 (TREM2) is an innate immune receptor that is expressed on the plasma membrane of microglia in the central nervous system (CNS) [1]. TREM2 is involved in key functions of microglia including phagocytosis, cytokine release, lipid sensing and microglia proliferation and migration [2–6]. *TREM2* mutations strongly increase the risk of developing Alzheimer's disease (AD) [7, 8] and other neurodegenerative diseases including frontotemporal dementia (FTD), Parkinson's disease and amyotrophic lateral sclerosis [9–12]. Furthermore, homozygous loss-of-function mutations in *TREM2* are sufficient to cause Nasu-Hakola disease (NHD) and FTD-like syndrome [13, 14]. Together, this suggests that abnormal TREM2 function plays an essential role across different neurodegenerative diseases.

TREM2 is a type-1 transmembrane protein that matures within the secretory pathway and its ectodomain is shed at the plasma membrane [2, 15]. Soluble TREM2 (sTREM2) accumulates in conditioned media of cultured cells and in biological fluids such as plasma and cerebrospinal fluid (CSF) [2, 16]. Shedding is mediated by ADAM10 and 17 C-terminal to histidine 157 [2, 15, 17–19]. Homozygous mutations causing NHD or FTD-like syndrome (such as p.T66M) retain misfolded TREM2 in the endoplasmic reticulum, preventing its maturation and its cleavage on the plasma membrane. Patients bearing these mutations have undetectable levels of sTREM2 in CSF and blood [2, 20, 21].

The fact that TREM2 is selectively expressed in microglia in the CNS and is associated with AD and neurodegeneration, let us hypothesize that sTREM2 in CSF may be a marker for microglia function and its response to Aβ and tau pathology and neurodegeneration. Specifically, sTREM2 may reflect the amount of signaling competent TREM2 on the surface of activated microglia. This idea is supported by the fact that the levels of sTrem2 in the brain of an Aβ mouse model correlate with TSPO small animal positron emission tomography (μPET) signal [22], a marker of microglial activation, and the fact that a *knock-in* mouse model bearing the Trem2 p.T66M mutation has decreased microglial activity [20].

We and others have previously reported changes in the levels of CSF sTREM2 in AD compared to controls [2, 21, 23–26]. Specifically, we found a disease-stage dependent increase in CSF sTREM2 peaking within the early symptomatic stages of late-onset AD [25]. In autosomal dominant AD (ADAD) assessed within the Dominantly Inherited Alzheimer Network (DIAN) project [26], we demonstrated that CSF sTREM2 was increased in mutation carriers compared to non-carriers five years before the estimated years from symptom onset (EYO), but with a considerable delay after the development of Aβ pathology, which emerged about 10-15 years earlier. Together, these studies suggest a complex association of CSF sTREM2 as a function of disease evolution, in which CSF sTREM2 dynamically changes as disease progresses and reaches its highest levels between the later asymptomatic and earlier symptomatic stages, when neurodegeneration has already started.

An important unanswered question in this regard concerns the association between CSF sTREM2 and primary pathologies including Aβ and tau deposition, as well as neurodegeneration during the course of AD. Therefore, we used herein the biomarker-based A/T/N classification system [27], which is the foundation of the recently proposed 2018 NIA-AA research Framework [28]. This classification system consists of three biomarker dimensions including the assessment of Aβ pathology (A), tau pathology (T), and neurodegeneration (N). In the present study, we investigated CSF sTREM2 levels at different AD biomarker-defined groups following the A/T/N classification and the clinical stage (as defined by the clinical dementia rating score, CDR) in participants of the well-characterized ADNI study. This approach allowed us to test the two main aims of this study. First, to assess the association of CSF sTREM2 with Aβ pathology and its downstream pathological processes (i.e. tau pathology and neurodegeneration). Second, to assess the changes on CSF sTREM2 that occur in the Alzheimer's *continuum* and hence replicate ours and others findings in the ADNI cohort [23–26, 29].

Methods

ADNI Participants and study design

This is a cross-sectional study in which CSF sTREM2 was measured in 1031 participants of the ADNI project. Among them, 4 individuals did not have the AD core biomarkers measurements and were further excluded from the analysis, rendering a study sample of 1027 subjects. The CSF sTREM2 measurements were uploaded to the ADNI database (http://adni.loni.usc.edu) on 16/03/2018

and the data used in this study was downloaded on 21/03/2018. The ADNI project (http://www.loni.usc.edu/) is a multicenter longitudinal study led by Principal Investigator Michael W Weiner with the main goal to develop and validate biomarkers for subject selection and as surrogate outcome measures in late-onset AD [30]. The institutional review boards (IRB) of all participating centers approved the procedures of the study and all participants or surrogates provided informed consent. Our local IRB (LMU) also approved the study.

Clinical classification

In line with the recently published 2018 NIA-AA "research framework" for the diagnosis of Alzheimer's disease [28], we assigned each ADNI participant in a group defined by its biomarker profile, as described by the A/T/N scheme [27], coupled with its cognitive status, as defined by the CDR score [31]. The A/T/N scheme comprises 3 biomarker groups: "A" refers to aggregated Aβ, "T" aggregated tau and "N" to neurodegeneration. Each biomarker group is binarizied in negative (-) or positive (+) based on whether their biomarkers are normal or abnormal. In the present study, we assigned "A+" to those individuals that had a CSF $A\beta_{1-42}$ < 976.6 pg/ml, "T+" to those individuals with P-tau_{181P} > 21.8 pg/ml and "N+" to those individuals with T-tau > 245 pg/ml. We merged the aggregated tau (T) and neurodegeneration (N) groups in order to decrease the number of groups to be compared. TN negative (TN-) was defined as having both the aggregated tau (T) and neurodegeneration (N) biomarkers in the normal range (T- and N-, that is P-tau_{181P} ≤ 21.8 pg/ml and T-tau ≤ 245 pg/ml). Participants were classified as TN positive (TN+) if either aggregated tau (T) or neurodegeneration (N) were abnormal (T+ or N+, that is P-tau_{181P} > 21.8 pg/ml or T-tau > 245 pg/ml). Only 5.4% of the individuals of the total differed between the T and N biomarkers groups.

The combination of the biomarker profile (A/T/N scheme) and the clinical status (CDR) rendered 12 different groups that are displayed in Table 1. We studied CSF sTREM2 in ADNI following two approaches. In a first one, we compared CSF sTREM2 between the different A/T/N categories within each clinical stage. In a second one, we attempted to model the course of AD with biomarker and clinical-based groups, similar to what was proposed by the 2011 NIA-AA criteria [32–34]. Thus, in this second approach, we compared the 'CDR = 0 A-/TN-' group (which corresponds to healthy controls) with those biomarker-based groups that fall into the Alzheimer's *continuum* category, that is: 'Preclinical AD A+/TN-', 'Preclinical AD A+/TN+', 'AD CDR = 0.5' and 'AD CDR = 1'. Since our aim was to study the Alzheimer's *continuum*, we excluded from this analysis those individuals that fall in the category of suspected non-Alzheimer's pathology (SNAP) [35–38] and those symptomatic individuals (CDR > 0) that do not have positive biomarkers for both Aβ deposition (CSF $A\beta_{1-42}$) and neurodegeneration/tau pathology (T-tau or P-tau_{181P}).

Table 1 Classification of ADNI participants based on the A/T/N framework and clinical stage

		Clinical stage (C)		
		CDR = 0 (cognitively unimpaired)	**CDR = 0.5** (very mild dementia)	**CDR = 1** (mild dementia)
Biomarker profile	**A-/TN-**	**Healthy controls** n = 122	n = 118	n = 2
	A+/TN-	**Preclinical AD A+/TN-** n = 52	n = 93	n = 15
	A+/TN+	**Preclinical AD A+/TN+** n = 45	**AD CDR = 0.5** n = 282	**AD CDR = 1** n = 80
	A-/TN+	n = 72	n = 94	n = 7
		suspected non-Alzheimer's pathology (SNAP)		

The ADNI participants were classified based on their clinical stage, as defined by the clinical dementia rating (CDR) score, and the biomarker-based A/T/N framework. The A/T/N framework comprises 3 biomarker groups: *A* Aβ pathology biomarker status, *T* tau pathology biomarker status, and *N* neurodegeneration biomarker status. Each of the biomarkers group have binarized into positive/abnormal (+) or negative/normal according the biomarkers cutoffs. T and N have been merged to simplify the classification and TN- indicates that both T and N are normal and TN+ indicates that T and/or N are abnormal.
The numbers shown here are excluding the *TREM2* mutation carriers and CSF sTREM2 outliers (as defined as values 3 standard deviations above or below the mean).
The colour indicates the different groups used for comparisons in the main text. Healthy controls (*n* = 122) are depicted in blue, the Alzheimer's *continuum* (*n* = 459) in red and the suspected non-Alzheimer's pathology (SNAP) group (*n* = 173) in green.
Bold text depicts the groups analysed when modelling the course of AD, namely 'healthy controls', 'Preclinical AD A+/TN -', 'Preclinical AD A+/TN+', 'AD CDR = 0.5' and 'AD CDR = 1'

Alzheimer's disease CSF core biomarkers and CSF sTREM2 measurements

In the present study, we used the AD CSF core biomarkers measurements performed with the Elecsys® total-tau CSF, the Elecsys® phosphotau(181P) CSF and the Elecsys® β-amyloid(1–42) CSF immunoassays on a cobas e 601 instrument [39, 40]. The data is available in the 'UPENNBIOMK9.csv' file in the ADNI database. These immunoassays are for investigational use only. They are currently under development by Roche Diagnostics and not commercially available yet. The analyte measuring ranges (lower technical limit to upper technical limit) of these assays are the following: 80 to 1300 pg/ml for total-Tau CSF, 8 to 120 pg/ml for phosphor-Tau(181P) CSF, and 200 to 1700 pg/ml for Elecsys® β-Amyloid(1-42) CSF immunoassays. The measuring range of the Elecsys® β-Amyloid(1-42) CSF immunoassay beyond the upper technical limit has not been formally established. Therefore use of values above the upper technical limit, which are provided based on an extrapolation of the calibration curve, is restricted to exploratory research purposes and is excluded for clinical decision making or for the derivation of medical decision points.

CSF sTREM2 measurements were done with a MSD platform-based assay, previously reported and validated [2, 25, 26]. A comprehensive description of the assay is shown in Supplementary methods (see Additional file 1: Supplementary methods). The CSF sTREM2 measurements are publicly available in the ADNI database.

Cell culture and transient transfection of HEK293T cells

HEK293T cells were cultured in DMEM with Glutamax I supplemented with 10 % (v/v) fetal calf serum (FCS) and 1 % penicillin/streptomycin. 24 hours after seeding, cells were transiently transfected with equal amounts of DNA coding for the different *TREM2* variants using Lipofectamine 2000 as the transfection reagent. *TREM2* variant constructs were generated by site-directed mutagenesis (Stratagene) of a *TREM2 wt* construct bearing N- and C-terminal HA and FLAG tags, respectively, as previously described [2]. All constructs were verified by DNA sequencing (GATC Biotech). We collected the conditioned media 48 hours after transfection. Cellular debris was removed by centrifugation at 4°C (13300 rpm, 20 min). Supernatants were subsequently frozen at -20°C until analyses were performed. Cell culture reagents were purchased from Thermo Fisher Scientific unless otherwise noted.

We measured the concentrations of HA-labeled TREM2 protein in the HEK293T conditioned media by two different ELISAs. First, sTREM2 concentrations were determined by the same ELISA used to measured sTREM2 in the human CSF samples, which includes a detection antibody against sTREM2 (see Additional file 1: Supplementary methods). Second, it was measured by a MSD platform-based including an antibody against the HA-tag. This second assay follows the same protocol as the first one but with the following modifications. The detection antibody is a monoclonal rat IgG anti-HA peptide sequence (YPYDVPDYA), clone 3F10 (Roche, Cat. No. 11 867 423 001; 100 ng/mL, 50 μL/well); the secondary antibody is a SULFO-TAG-labeled goat polyclonal anti-rat IgG antibody (MSD, Cat. no. R32AH-1; 0.5 μg/mL, 25 μL/well). The samples were diluted 1:50 and 1:100 in assay buffer [0.25% BSA and 0.05% Tween 20 in PBS (pH = 7.4)], supplemented with protease inhibitors (Sigma; Cat. no. P8340) and measured in duplicates for each dilution. We acquired the electrochemiluminescence response values using the MESO QuickPlex SQ 120. We compared the signal of the sTREM2 ELISA with that of the HA-tag assay for each of the TREM2 rare variants. The percentage between these two assays renders a relative affinity of the sTREM2 ELISA to each of the TREM2 rare variants in relation to its respective HA-tag control.

Statistical analysis

CSF sTREM2 did not follow a normal distribution (Kolmogorov-Smirnov test: $P < 0.0001$) and were hence $\log_{10}$-transformed. After transformation, CSF sTREM2 followed a normal distribution as assessed by Kolmogorov-Smirnov test ($P = 0.200$) and visual inspection of the histogram. All the statistical analysis described in this study are performed with the $\log_{10}$-transformed values.

A one-way analysis of covariance (ANCOVA) was conducted to determine statistically significant differences on CSF sTREM2 between *TREM2* rare variants carriers and the non-carriers' individuals adjusting for the effect of age, followed by a Bonferroni corrected *post hoc* pairwise comparison. Only those groups of *TREM2* rare variants carriers that comprise more than 1 subject were included in the analysis.

The following analyses were conducted excluding outliers' values of CSF sTREM2, defined as values differing 3 standard deviations from the mean. There were 5 outliers: 2 subjects classified as 'Preclinical AD A+TN-' (1 a *TREM2* rare variant carrier and 1 a non-carrier), 1 classified as 'CDR = 0.5 A+TN-' (*TREM2* rare variant carrier), 1 classified as 'AD CDR = 0.5' (*TREM2* rare variant carrier), 1 classified as 'AD CDR = 1' (non-carrier). Including or excluding these outliers do not change the findings of this study.

To study the association of CSF sTREM2 with demographic and genetic data, we computed a linear regression model with CSF sTREM2 as an outcome variable and age, gender and *APOE* ε4 status as fixed effects. Since only age showed to be a significant predictor of

CSF sTREM2, the following analyses were conducted including only age as a covariate.

To test the differences in CSF sTREM2 across biomarker profiles in the A/T/N framework, we applied a one-way ANCOVA including age as covariate, followed by Bonferroni corrected *post hoc* pairwise comparisons. A similar approach was used to test whether CSF sTREM2 changes across the Alzheimer's *continuum*. These analyses were performed including or excluding individuals carrying a *TREM2* rare variant and yielded similar results.

Finally, we studied the association between CSF sTREM2 and each of the CSF core biomarkers for AD (T-tau, P-tau$_{181P}$, Aβ_{1-42}) with a multiple linear regression adjusted for age. The analysis was conducted separately in the healthy controls, Alzheimer's *continuum* and SNAP groups. We performed the analysis both including or excluding outliers (defined as AD CSF core biomarkers 3 standard deviations below or above the group mean) in order to exclude that the associations were driven by extreme values. The analysis with and without outliers rendered similar results. For CSF Aβ_{1-42}, the analyses were performed using both the truncated values at the upper technical limit and the exploratory measurements available based on the extrapolation of the calibration curve. In the main text, we report the results using the extrapolated measurements, but using the truncated ones yielded similar results.

Statistical analysis was performed in SPSS IBM, version 20.0, and the free statistical software R (http://www.r-project.org/). Figures were built using GraphPad Prism or free statistical software R. All tests were 2-tailed, with a significance level of $\alpha = 0.05$.

Results

Association of CSF sTREM2 with genetic and demographical data

We studied a total of 1027 participants of the ADNI study. The demographical and clinical characteristics of the whole study population are described in Table S1 (see Additional file 1). Among the participants studied, 43 (4.2 %) had a known *TREM2* rare variant (see reference [41] for a comprehensive review of the pathogenicity of each variant). The overall mean levels of CSF sTREM2 of these individuals (M = 3913 pg/ml, SD = 3548, n = 43) were significantly lower than the rest of ADNI participants without a *TREM2* rare variant (M = 4136 pg/ml, SD = 2171, n = 984; $F_{1,1024} = 6.77$, $P = 0.009$, $\eta_p^2 = 0.007$; Table 2, Fig. 1) in a one-way ANCOVA adjusted for age. However, CSF sTREM2 varied considerably between *TREM2* variants ($F_{4,1019} = 8.79$, $P < 0.0001$, $\eta_p^2 = 0.033$) and Bonferroni's *post hoc* comparisons test indicated that the p.R47H variant [7–12] had significantly higher CSF sTREM2 ($P = 0.003$) and the p.L211P variant [42, 43] significantly lower CSF sTREM2 ($P = 0.002$) than non-carriers. No differences in CSF sTREM2 were found between individuals with a p.R62H [44, 45] and the p.D87N [7] variants and the non-carriers. There was a single subject carrying both a p.D87N and p.R62H variants, and another single subject carrying a p.H157Y variant, which were not included in the statistical analysis. However, it is worth noting that the subject carrying a p.H157Y *TREM2* rare variant had relatively high CSF sTREM2 (Table 2, Fig. 1), an observation that agrees with our previous findings that the p.H157Y variant, which is located exactly at the cleavage site, increases shedding of TREM2 [17]. Given that

Table 2 Demographic and clinical characteristics of the individuals carrying a *TREM2* rare variant

	Non-carriers (*n* = 984)	p.R62H (*n* = 20)	p.R47H (*n* = 7)	p.L211P (*n* = 11)	p.D87N (*n* = 3)	p.R62H/D87N (*n* = 1)	p.H157Y (*n* =1)
Age, y	73.1 (7.35)	74.7 (6.47)	73.5 (11.3)	72.8 (4.36)	72.7 (6.17)	66.4	73.1
Female, n (%)	430 (43.7)	11 (55.0)	3 (42.9)	6 (54.5)	0	0	1
APOE ε4 carriers, n (%)	467 (47.5)	8 (40.0)	5 (71.4)	2 (18.2)	2 (66.7)	0	0
Education, y	16.0 (2.78)	15.7 (2.39)	15.6 (2.07)	14.6 (2.54)	17.0 (2.65)	15.0	18.0
CSF biomarkers (pg/ml)							
T-tau	289 (136)	322 (140)	353 (125)	231 (119)	299 (100)	116	214
P-tau$_{181P}$	27.9 (14.9)	30.9 (15.6)	36.4 (15.8)	22.2 (12.7)	27.9 (11.5)	9.92	18.1
Aβ_{1-42}	982 (457)	1073 (437)	874 (454)	1246 (515)	944 (670)	925	1700
sTREM2	4136 (2171)	3418 (1786)	8790 (6136)	2386 (1390)	1981 (244)	518	5642
Associated diseases	na	AD	AD, FTD, PD, ALS	AD, FTD	AD	AD	AD
References	na	[44, 45]	[7–12]	[42, 43]	[7]	[7, 44, 45]	[56]

Data are expressed as mean and standard deviation (SD) or number (n) and percentage (%), as appropriate.
Abbreviations: *Aβ$_{1-42}$* amyloid-β 42, *AD* Alzheimer's disease, *ALS* amyotrophic lateral sclerosis, *APOE* apolipoprotein E, *CSF* cerebrospinal fluid, *FTD* frontotemporal dementia, *na* non-applicable, *PD* Parkinson's disease, *P-tau$_{181P}$* tau phosphorylated at threonine 181, *T-tau* total tau, *y* years.

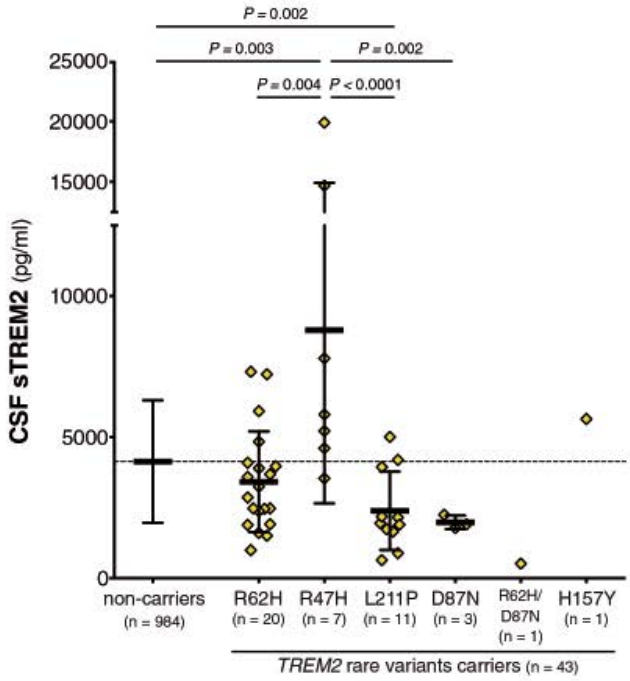

Fig. 1 CSF sTREM2 in ADNI participants carrying a *TREM2* rare variant. Scatter plot representing the levels of CSF sTREM2 in carriers of a *TREM2* rare variant, compared to the non-carriers ADNI participants. Solid bars represent the mean and the standard deviation (SD). *P*-values were assessed by a one-way ANCOVA adjusted for age, followed by Bonferroni corrected *post hoc* pairwise comparisons between the *TREM2* variants carriers' groups and the non-carriers. We did not include in the comparison those rare variants with only one subject (p.R62H/p.D87N and p.H157Y).

TREM2 rare variants may influence CSF sTREM2 (as described here and in [24]), all the following analyses are excluding participants carrying these rare variants. Nevertheless, including the *TREM2* rare variants carriers did not change the results. In order to test whether the differences in CSF sTREM2 among *TREM2* rare variants are influenced by differences in the antibody affinity to the mutant sTREM2, we transfected HEK293T cells with an epitope tagged *wild type* (*wt*) and mutated TREM2 and measured sTREM2 released in the media with the same ELISA used for the quantification of CSF sTREM2 and with an ELISA using an antibody against the epitope tag (Additional file 1: Figure S1). This revealed that the p.R47H, p.R62H and p.H157Y *TREM2* rare variants were detected with a slightly reduced efficiency in our ELISA; therefore, the increased levels of CSF sTREM2 found in subjects bearing the p.R47H rare variants are even slightly underestimated. On the other hand, the p.L211P *TREM2* rare variants were detected efficiently, independently of their individual amino acid exchanges. However, the p.D87N *TREM2* rare variant was detected with significant less affinity in our ELISA than using the antibody against the epitope tag. Thus, the decreased CSF sTREM2 found in the p.D87N rare variant should be interpreted with caution.

In the sample excluding the *TREM2* rare variants carriers and TREM2 outliers' values (n = 982, see methods section), we first assessed which demographic and genetic variables are associated with CSF sTREM2 (descriptives summarized in Table 3). Consistent with previous results [21, 23–26], CSF sTREM2 levels were associated with age (β = +0.275, P < 0.0001, η_p^2 = 0.073), but not with gender ($F_{1,978}$ = 0.029, P = 0.866, η_p^2 = 0.00003) or *APOE* ε4 status ($F_{1,978}$ = 0.099, P = 0.753, η_p^2 = 0.0001). Consequently, all further analysis included age as a covariate, but not gender or *APOE* ε4 status.

Differences of CSF sTREM2 within the A/T/N classification of AD

In order to assess the impact of Aβ deposition or the downstream processes of the amyloid cascade (i.e. tau pathology and neurodegeneration), we applied the recently proposed A/T/N classification framework of AD,

Table 3 Demographic and clinical characteristics of the sample excluding the individuals carrying a *TREM2* rare variant

	CDR = 0 (n = 291)				CDR = 0.5 (n = 587)				CDR = 1 (n = 104)			
	A-/TN- (*n* = 122)	A+/TN- (*n* = 52)	A+/TN+ (*n* = 45)	A-/TN+ (*n* = 72)	A-/TN- (*n* = 118)	A+/TN- (*n* = 93)	A+/TN+ (*n* = 282)	A-/TN+ (*n* = 94)	A-/TN- (*n* = 2)	A+/TN- (*n* = 15)	A+/TN+ (*n* = 80)	A-/TN+ (*n* = 7)
Age, y	72.5 (5.50)	73.5 (5.95)	76.3 (5.46)	74.6 (6.56)	69.9 (7.56)	72.6 (7.74)	73.3 (7.08)	73.2 (8.16)	89.2 (1.63)	76.3 (6.05)	74.1 (9.28)	79.8 (8.02)
Female, n (%)	58 (47.5)	24 (46.2)	24 (53.3)	40 (55.6)	55 (46.6)	21 (22.6)	119 (42.2)	42 (44.7)	0 (0)	5 (33.3)	40 (50.0)	2 (28.6)
APOE ε4 carriers, n (%)	17 (13.9)	20 (38.5)	27 (60.0)	15 (20.8)	24 (20.3)	47 (50.5)	216 (76.6)	32 (34.0)	0 (0)	8 (53.3)	59 (73.8)	1 (14.3)
Education, y	16.4 (2.79)	16.0 (2.65)	16.5 (2.55)	16.4 (2.57)	16.0 (2.68)	16.1 (2.97)	15.9 (2.90)	15.9 (2.66)	17.0 (0)	16.2 (2.51)	15.1 (2.80)	15.0 (2.08)
CSF biomarkers, pg/ml*												
T-tau	185 (32.2)	167 (40.5)	332 (79.3)	322 (71.9)	183.4 (38.2)	173 (40.0)	381 (136)	338 (111)	234 (6.08)	188 (38.8)	393 (136)	489 (221)
P-tau$_{181P}$	16.2 (2.88)	15.4 (4.07)	33.3 (9.09)	28.9 (7.43)	15.8 (3.32)	15.9 (4.14)	38.9 (14.7)	31.6 (13.7)	19.3 (1.20)	17.4 (3.53)	39.2 (15.0)	43.2 (22.9)
Aβ$_{1-42}$	1456 (223)	723 (196)	717 (168)	1538 (235)	1428 (247)	632 (196)	634 (168)	1427 (290)	1330 (14.9)	538 (185)	570 (159)	1484 (290)
sTREM2	3741 (1690)	2835 (1524)	4839 (2240)	5378 (2147)	3436 (1754)	2791 (1292)	4441 (2211)	5478 (2370)	5660 (2008)	3051 (1128)	3967 (2000)	7619 (3539)

Data are expressed as mean and standard deviation (SD) or number (n) and percentage (%), as appropriate.
*The CSF core biomarkers measurements were performed using the electrochemiluminiscence immunoassays Elecsys Total-tau CSF, phosphor-tau(181P) CSF and β-amyloid(1-42) CSF, which have an upper technical limit of 1300 pg/ml (T-tau), 120pg/ml (P-tau181P) or 1700 pg/ml (Aβ$_{1-42}$). The values above these limits were truncated to the respective upper technical limit.
Abbreviations: *A* Aβ pathology biomarker status, *Aβ$_{1-42}$* amyloid-β 42, *APOE* apolipoprotein E, *CDR* clinical dementia rating, *CSF* cerebrospinal fluid, *N* neurodegeneration biomarker status, *P-tau$_{181P}$* tau phosphorylated at threonine 181, *T* tau pathology biomarker status, *T-tau* total tau, *y* year

which proposes 3 binary biomarker groups [27]: (1) aggregated Aβ (A+/A-), (2) aggregated tau (T+/T-) and (3) neurodegeneration (N+/N-). Given that CSF T-tau and CSF P-tau$_{181P}$ were highly correlated, we merged the tau (T) and neurodegeneration (N) groups. 'TN-' profile was defined as both CSF P-tau$_{181P}$ and T-tau within the normal range, whereas 'TN+' was defined as abnormal levels of CSF P-tau$_{181P}$ or T-tau. Thus, we compared 4 different biomarker profiles within each clinical stage, namely: (1) A-/TN-, (2) A+/TN-, (3) A+/TN+ and (4) A-/TN+.

Within the CDR = 0 group (i.e. cognitively normal individuals), a one-way ANCOVA showed a significant difference between the four biomarker profiles after adjusting for the effect of age ($F_{3,286}$ = 21.3, P < 0.0001, η_p^2 = 0.183). A Bonferroni *post hoc* test revealed that the A+/TN- profile had significant lower CSF sTREM2 compared to either other biomarker profile (Fig. 2). Only the A-/TN+ profile had significant higher CSF sTREM2 compared to the normal biomarkers profile (i.e. A-/TN-).

Within the CDR = 0.5 group (i.e. very mild dementia), there was also a significant difference between the four biomarker profiles ($F_{3,582}$ = 40.7, P < 0.0001, η_p^2 = 0.173) and the A+/TN- profile had also the significantly lowest CSF sTREM2 compared to either other biomarker profile (Fig 2). Both the A+/TN+ and the A-/TN+ biomarker profiles had significant higher CSF sTREM2 compared to the A-/TN- profile (Fig. 2). The CDR = 1 group did not yield a sufficient number of subjects per A/T/N profile to allow for a group comparison.

We repeated the former analysis also including the individuals with *TREM2* rare variants (n = 43; demographics in Table S1, see Additional file 1) and this did not change our conclusions derived from the main analysis (Fig. 2).

We also repeated the same analysis classifying the participants based only on their Aβ pathology (A; CSF Aβ$_{1-42}$) and tau pathology status (T; CSF P-tau$_{181P}$), that is A/T classification, or based only on their Aβ pathology (A; CSF Aβ$_{1-42}$) and neurodegeneration status (N; CSF T-tau), that is A/N classification. The results are shown

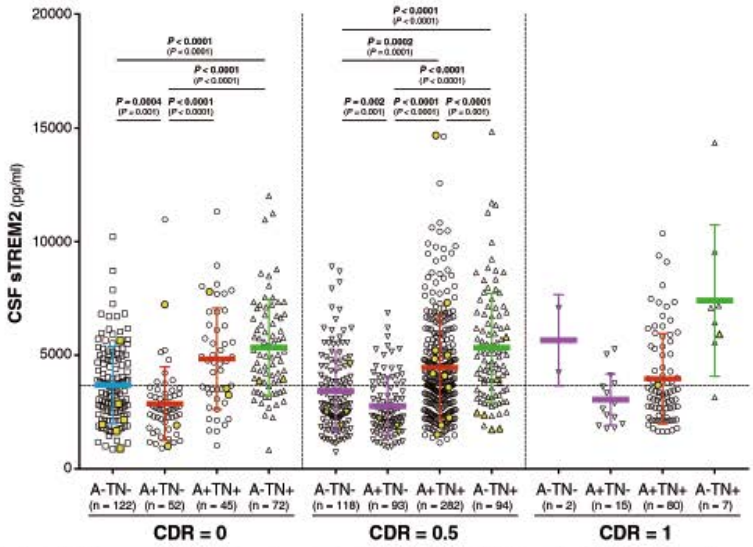

Fig. 2 CSF sTREM2 in the A/T/N framework. Scatter plot depicting the levels of CSF sTREM2 for each of the four biomarker profiles, as defined by the A/T/N framework, coupled with clinical staging, as defined by CDR. The biomarkers groups T (tau pathology) and N (neurodegeneration) were merged in order to reduce the number of groups to compare. The CDR = 1 stage includes some biomarker profiles will low number of subjects, which precludes performing statistical analysis. They are still shown in the figure for sake of completeness. Each biomarker category is represented in a different colour: healthy controls are depicted in blue, Alzheimer's *continuum* category in red, SNAP category in green, and purple depicts biomarker profiles not assigned in a specific category in the present study. The analysis reported in the main text was conducted excluding the *TREM2* rare variants carriers, the *P*-values are reported in bold, and the number of individuals (n) per group indicated. Including these carriers (depicted in yellow) rendered similar results (*P*-values reported between brackets). Solid bars represent the mean and the standard deviation (SD). *P*-values were assessed by a one-way ANCOVA adjusted for age, followed by Bonferroni corrected *post hoc* pairwise comparisons. Abbreviations: A: Aβ pathology biomarker status; AD: Alzheimer's disease; CDR: clinical dementia rating; CSF: cerebrospinal fluid; N: neurodegeneration biomarker status; SNAP: suspected non-Alzheimer's pathology; T: tau pathology biomarker status.

in Figure S2 (see Additional file 1) and they are similar to those shown with the A/TN classification of the main text. Thus, we conclude that the pathological processes that are downstream of Aβ pathology, both tau pathology and neurodegeneration, are associated with increased CSF sTREM2.

CSF sTREM2 changes across the Alzheimer's *continuum*

Next we asked if CSF sTREM2 changes during the course of the disease as previously described in our study on late onset-AD [25]. We modeled the evolution of AD comparing the biomarker-defined groups (Table 1) that reflect the temporary course of late-onset AD, similar to what was proposed by the previous 2011 NIA-AA diagnostic criteria [32–34]. Thus, we compared the 'healthy controls' group (highlighted in blue in Table 1 and corresponding to the the 'CDR = 0 A-/TN-' group), with those belonging to the Alzheimer's *continuum* (highlighted in red in Table 1), which included: 'Preclinical AD A+/TN-','Preclinical AD A+/TN+', 'AD CDR = 0.5' and 'AD CDR = 1'. A one-way ANCOVA revealed that CSF sTREM2 was significantly different between groups after adjusting for the effect of age ($F_{4,575}$ = 11.5, P < 0.0001, η_p^2 = 0.074). A *post hoc* analysis using the Bonferroni criterion for significance indicated that the average CSF sTREM2 was significantly higher in the 'AD CDR = 0.5' group than in the 'healthy controls' and 'Preclinical AD A+TN-' groups (P = 0.034 and P < 0.0001, respectively; Fig. 3). Similar results were obtained when the individuals carrying a *TREM2* rare variant were included (Fig. 3). Thus, these results replicate our and other groups previous findings of increased CSF sTREM2 in early symptomatic stages of late-onset AD in an independent sample [23–25, 29].

CSF sTREM2 is associated with T-tau and P-tau but not $A\beta_{1\text{-}42}$

Finally, we studied the associations of CSF sTREM2 with each of the CSF core biomarkers of AD, that is T-tau, P-tau_{181P} and $A\beta_{1\text{-}42}$, in linear regression models adjusted for age. The associations were tested separately in three groups based on their biomarker profile (see Table 1): (1) healthy controls, (2)

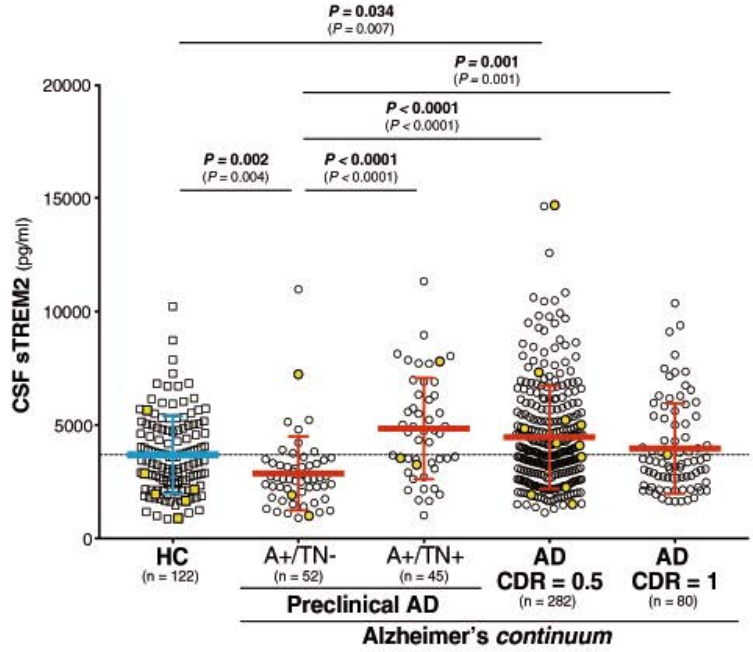

Fig. 3 CSF sTREM2 across the Alzheimer's *continuum*. Scatter plot showing the levels of CSF sTREM2 in healthy controls (depicted in blue) and the different stages of the Alzheimer's *continuum* (depicted in red). The main analysis was conducted excluding the *TREM2* rare variants carriers, the *P*-values are reported in bold, and the number of individuals (n) per group indicated. Including these carriers (depicted in yellow) rendered similar results (*P*-values reported between brackets) *P*-values were assessed by a one-way ANCOVA adjusted for age, followed by Bonferroni corrected *post hoc* pairwise comparisons. Abbreviations: A: Aβ pathology biomarker status; AD: Alzheimer's disease; CDR: clinical dementia rating; CSF: cerebrospinal fluid; N: neurodegeneration biomarker status; SNAP: suspected non-Alzheimer's pathophysiology; T: tau pathology biomarker status.

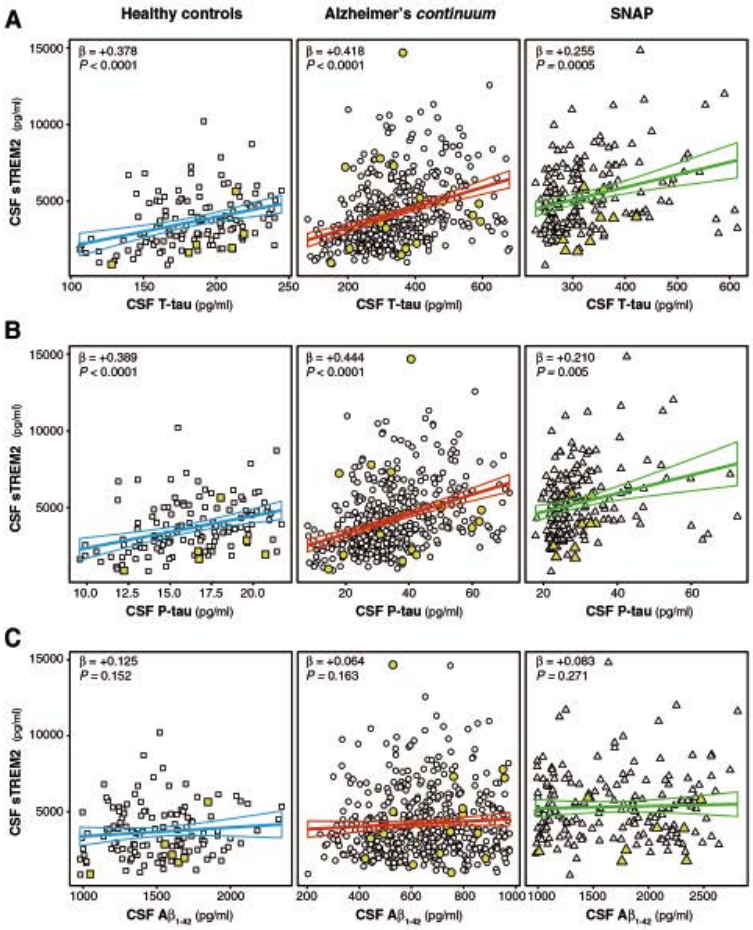

Fig 4. Association of CSF sTREM2 and AD core CSF biomarkers. Scatter plots representing the associations of CSF sTREM2 with each of the AD CSF core biomarkers: T-tau (**a**), P-tau$_{181P}$ (**b**), and Aβ$_{1-42}$ (**c**) in three different groups defined by the biomarker profile: healthy controls (blue), Alzheimer's *continuum* (red) and SNAP groups (green). The solid lines indicate the regression line and the 95% confidence interval for each of the groups. The standardized regression coefficients (β) and the *P*-values are shown and were computed using a linear model adjusting for age, and are conducted excluding the outliers values. Including them, did not change the conclusions (see Additional file 1: Table S2). We also performed the analysis including the participants carrying a *TREM2* rare variant (depicted in yellow) and the results were similar (see Additional file 1: Table S3). Abbreviations: Aβ$_{1-42}$: amyloid-beta 42; T-tau: total tau; P-tau: tau phosphorylated at Threonine 181; SNAP: suspected non-Alzheimer's pathology.

individuals of the Alzheimer's *continuum* and (3) in the SNAP groups. Consistent with previous findings, CSF sTREM2 is associated with T-tau and P-tau$_{181P}$ in the three groups studied (Fig. 4). In contrast, no significant associations were found between CSF sTREM2 and Aβ$_{1-42}$ (Fig. 4). Including the CSF biomarkers outliers (see Additional file 1: Table S2), or including the individuals carrying a *TREM2* rare variants (see Additional file 1: Table S3), did not change our findings.

Discussion

In the present study, we assessed the microglial-activity marker CSF sTREM2 within the early phases of AD. To this end, we applied the biomarker-based A/T/N classification in combination with clinical staging [27]. The use of this classification system enabled us to unravel the effect of Aβ pathology and its downstream processes (i.e. tau pathology and neurodegeneration) on the levels of CSF sTREM2. Interestingly, they are differentially associated with CSF sTREM2. While pure Aβ deposition (as

defined here as low CSF $A\beta_{1-42}$) is associated with decreased CSF sTREM2, tau pathology or neurodegeneration (as defined here as increased CSF P-tau_{181P} or CSF T-tau, respectively) are associated with an increase in CSF sTREM2. The higher CSF sTREM2 in the SNAP groups confirm that CSF sTREM2 rises with neurodegeneration without Aβ pathology.

Moreover, we show that the levels of CSF sTREM2 differ between *TREM2* variants and they are increased in the p.R47H *TREM2* variant compared to non-carriers, but decreased in the p.L211P variant and remained unchanged in the p.R62H variant, which is consistent with previous reports [24]. Increased levels of sTREM2 in the p.R47H variant were somewhat surprising; however, very little is known if that variant affects proteolytic processing of TREM2. In contrast, the increased levels of the p.H157Y variant is in line with our previous findings in cultured cells demonstrating that this variant increased shedding of TREM2 and therefore decreased TREM2 dependent phagocytosis [17]. A word of caution should be noted with the p.D87N variant, because the lower levels could be due, at least partially, to the lesser affinity of the antibody to this variant.

Among the strengths of this study are the large and well-characterized sample size and the use of a reliable assay to measure sTREM2. Yet, this study has some limitations. First, this is a cross-sectional study and the results need to be confirmed in a longitudinal setting. Second, we used the CSF biomarkers to classify the ADNI participants in the A/T/N classification. Although the role of CSF biomarkers in AD is well-established and a complete A/T/N characterization is possible only with CSF biomarkers, CSF T-tau may not necessarily reflect neurodegeneration but could result from physiological production of tau [46]. Here, we found CSF P-tau_{181P} and CSF T-tau to be highly correlated (only 5.4% of all the ADNI participants had a discrepant T and N biomarker group), and consequently we merged the "T" (tau pathology) and "N" (neurodegeneration) groups. Importantly, the TN- group had normal levels of both T-tau and P-tau_{181P}, which reasonably ensure that other comorbidities that may cause neural injury (and hence microglial activation) are excluded. A plethora of other biofluid and neuroimaging markers (i.e. Aβ and tau PET, blood and CSF neurofilament light protein, anatomic MRI and FDG-PET) are in principle applicable for the implementation of the A/T/N classification, and future studies using these biomarkers are needed to confirm our results.

The A/T/N classification used herein is a descriptive biomarker-based classification that does not assume any temporal sequence of events in AD and is independent of the clinical stage of the disease. By applying this classification framework, we found an unexpected observation, namely a decrease of CSF sTREM2 in individuals with evidence of Aβ pathology but without signs of tau pathology or neurodegeneration. We did not observe this finding in previous studies, probably due to the low number of participants in the preclinical stage of late-onset AD and because we did not apply the A/T/N classification [25]. Noteworthy, we previously observed in ADAD that CSF sTREM2 was lower in ADAD mutation carriers than in non-carriers at very early stages (EYO < -15; CSF T-tau becomes significantly increased in mutation carriers at EYO = -15), yet statistically non-significant [26]. In contrast, we show here that CSF sTREM2 increases as soon there are signs of we show here that CSF sTREM2 increases, both in the context of AD (that is with co-occurrence of Aβ pathology) or in the SNAP patients (where there is no Aβ underlying pathology). Consistent with these findings, CSF sTREM2 is distinctly associated with CSF T-tau and CSF P-tau_{181P}, but not with CSF $A\beta_{1-42}$. A word of caution is needed in the SNAP category, given that this is an heterogenous group that most likely exhibits a non-AD related neurodegeneration. Well-powered future studies should address how CSF sTREM2 changes in neurodegenerative diseases different from AD.

The flexibility of this new classification framework also enabled us to model the natural history of AD and confirm in the ADNI study our previous findings in participants of several European memory clinics [25] who were classified using the 2011 NIA-AA criteria [32–34, 36]. Herein, we demonstrate that, after the initial decrease of CSF sTREM2 in the 'Preclinical AD A+/TN-' group, CSF sTREM2 rise is the 'Preclinical AD A+/TN+'group and in the early symptomatic stage (CDR = 0.5) of AD, albeit only statistically significant in the latter group. These findings therefore replicate our [25, 26] and other groups [23, 24, 29] previous findings in which an increase in CSF sTREM2 in early symptomatic AD was observed.

The mechanism underlying the dynamic changes of CSF sTREM2 throughout the course of the disease still need to be investigated. Several studies have consistently demonstrated that microglia upregulate TREM2 expression in AD mouse models and in human AD brains [3, 47, 48]. Moreover, detailed transcriptomics studies that investigated microglia in mouse models of AD and neurodegeneration showed that TREM2 is upregulated in the disease-associated microglia (DAM) [1, 49–53]. This is consistent with the finding of increased CSF sTREM2 in stages downstream of Aβ accumulation, that is when tau pathology and/or neurodegeneration occur and microglia may adopt their disease associated molecular signature. We were surprised, however, by the observation of an initial CSF sTREM2 decrease in the only Aβ-pathology stage, which corresponds to the earliest stage of the disease. The possible mechanisms behind this observation are still elusive. However, it has been described that

microglia are activated in two steps with an initial TREM2-independent process followed by a TREM2-dependent process [49]. The CSF sTREM2 increase observed following the initially low levels may reflect the second step of activation. An alternative explanation would be that microglia initially forms a barrier around plaques [54, 55] and the sTREM2 released by microglia is retained within the plaque, until the barrier fails, and subsequent neural injury starts. Finally, it could also be argued that subjects with low TREM2-function (and hence lower CSF sTREM2 levels) are more prone to experience an accelerated early amyloidogenesis (Parhizkar *et al.* Nat. Neursci *in press*) and are therefore overrepresented in the Preclinical AD A +TN- group. Nevertheless, we are cognizant of the fact that this is an observational study and the findings reported herein do not elucidate precise mechanisms. Further work with longitudinal data is needed to address whether the stage-dependent changes in CSF sTREM2 predict a better or worse clinical outcome.

In conclusion, the present study represents the first attempt to study CSF sTREM2 based on the A/T/N classification framework. We demonstrate in the ADNI cohort that the increase in CSF sTREM2 which occurs in early stages parallels the increase in biomarkers of tau pathology and neurodegeneration. In contrast, Aβ deposition in the absence of tau deposition and neurodegeneration is associated with lower CSF sTREM2.

Additional file

Additional file 1: CSF sTREM2 measurement. **Figure S1.** Effects of rare *TREM2* variants on antibody affinities. **Figure S2.** CSF sTREM2 in the A/T and in the A/N classifications. **Table S1.** Demographic and clinical characteristics of the entire sample. **Table S2.** Associations of CSF sTREM2 with AD CSF core biomarkers including the biomarkers outliers. **Table S3.** Associations of CSF sTREM2 with AD CSF core biomarkers including subjects carrying a TREM2 rare variant. (DOCX 1992 kb)

Abbreviations

Aβ: Amyloid β-peptide; AD: Alzheimer's disease;; ADAD: Autosomal-dominant Alzheimer's disease; CDR: Clinical dementia rating score; CNS: Central nervous system; CSF: Cerebrospinal fluid; EYO: Estimated years from symptom onset; SNAP: Suspected non-Alzheimer's pathology; sTREM2: Soluble triggering receptor expressed on myeloid cells 2; TREM2: Triggering receptor expressed on myeloid cells 2

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Availability data and materials

The dataset supporting the conclusions of this article is available in the ADNI site, http://adni.loni.usc.edu/.

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Authors' contributions

MSC, EMR, GK, AC, KF, BN, EE, YD and LP performed the experiments. MSC, MAC, NF, YD, ME and CH analyzed and interpreted the data. KS and EMR performed the cell culture experiments. YD, LP, CMK and CC were involved in extracting the genetic data. JL, LS, JQT, MW contributed with patient samples and/or data. MSC, ME and CH designed the study and wrote the manuscript. All authors critically reviewed and approved the final manuscript.

Ethics approval and consent to participate

The study was approved by the Ludwig-Maximilians Universität München institutional review board (IRB), as well as the IRB of all participating centers in ADNI.

Consent for publication

Not applicable.

Competing interests

CH collaborates with DENALI Therapeutics and received speakers honoraria from Novartis and Roche. KS collaborates with DENALI. JL reports to receive consulting fees from Aesku, Axon Neuroscience and Ionis Pharmaceuticals, speakers' fees from Bayer Vital and the Willi Gross and non-financial support from Abbvie, outside the submitted work. CC receives research support from Biogen, EISAI, Alector and Parabon, and is a member of the advisory board of ADx Healthcare. The funders of the study had no role in the collection, analysis, or interpretation of data; in the writing of the report; or in the decision to submit the paper for publication. The remaining authors declare that they have no competing of interest.

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