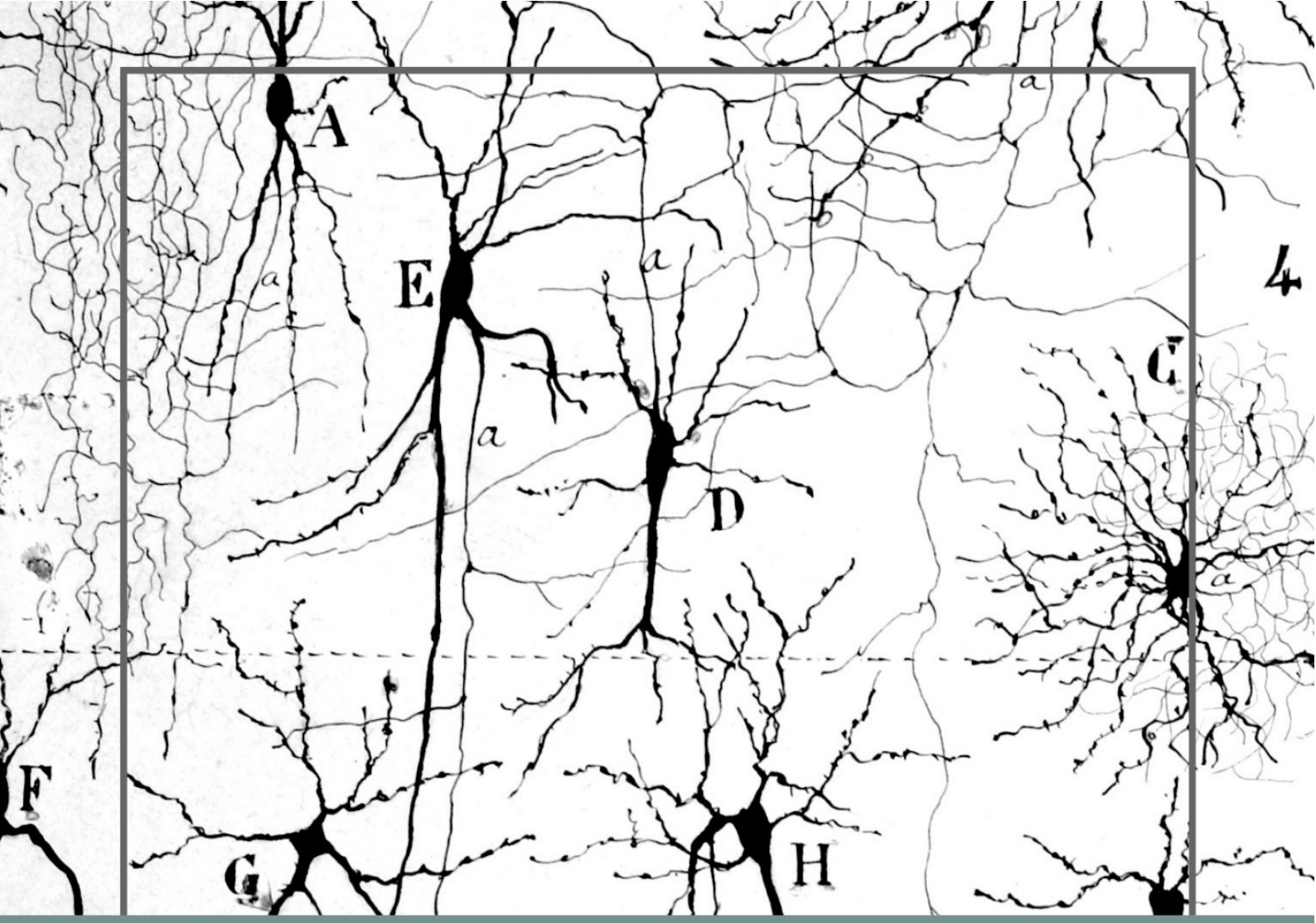
Universitat Autònoma de Barcelona

Mixed neurodegenerative dementia: the coexistence of dementia with Lewy bodies and Alzheimer´s disease Expanding our knowledge about the interaction of brain proteinopathies

Carla Abdelnour Ruiz



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Cover image: drawings of neocortical neurons from Santiago Ramón y Cajal.

DOCTORAL THESIS

Mixed neurodegenerative dementia:

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Expanding our knowledge about the interaction of brain proteinopathies

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“We are a way for the cosmos to know itself”

Carl Sagan

Acknowledgements

I believe in making the world a better place, and I have chosen to make my contribution by trying to solve one of the big problems we face today: dementia. But like with any big problem, it cannot be solved by one individual: it needs the collaboration and hard work of a team.

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Abbreviations

Aβ42:	Amyloid-β42
AD:	Alzheimer´s disease
ALS:	Amyotrophic lateral sclerosis
APOE:	Apolipoprotein E
APP:	Amyloid precursor protein
AT(N):	Amyloid-β deposition, pathologic tau, and neurodegeneration
CSF:	Cerebrospinal fluid
CT:	Computarized tomography
DAT:	Dopamine transporter
DaTSCAN:	Dopamine transporter single photon emission computerized tomography
DLB:	Dementia with Lewy bodies
DMTs:	Disease modifying treatments
E-DLB:	European dementia with Lewy bodies Consortium
EEG:	Electroencephalography
FAMD:	Factorial analysis of mixed data
FDG-PET:	Fluodeoxiglucose positron emission tomography
FTD:	Frontotemporal dementia
FUS:	Fused in sarcoma
GBA:	Glucosylceramidase Beta
GCA-F:	Global cortical atrophy-frontal subscale
GRN:	Progranulin
GWAS:	Genome wide association study
LME:	Linear mixed effect
LPC:	Lewy pathology consensus criteria
MAPT:	Microtubule-associated protein tau gene
MCI:	Mild cognitive impairment
MIBG:	Iodine-123 metaiodobenzylguanidine myocardial scintigraphy
MMSE:	Mini Mental State Examination
MoCA:	Montreal Cognitive Assessment

MRI:	Magnetic resonance imaging
MSA:	Multisystem atrophy
MTA:	Medial temporal lobe atrophy
NFT:	Neurofibrillary tangles
NIA-AA:	National Institute on Aging-Alzheimer's Association
OOB-EER:	Out-of-the-bag estimated error rate
p-tau:	Phosphorylated tau at threonine 181
PA	Posterior atrophy
PD:	Parkinson´s disease
PDD:	Parkinson´s disease dementia
PET:	Positron emission tomography
PSEN1:	Presenilin 1
PSEN2:	Presenilin 2
PSG:	Polisomnography
PSP:	Progressive supranuclear palsy
RBD:	Rapid eye movement sleep behavior disorder
REM:	Rapid-eye-movement
RT-QuIC:	Real-Time Quaking-Induced Conversion
SNCA:	Synuclein Alpha
SPECT:	Single photon emission computerized tomography
T-tau:	Total tau
TDP-43:	TAR DNA-binding protein 43
ThT:	Thioflavin T
UPDRS:	Unified Parkinson disease rating scale
UPS:	Ubiquitin proteasome
VH:	Visual hallucinations
WMH:	White matter hyperintensities

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Abstract

Dementia with Lewy bodies (DLB) is the second most common cause of neurodegenerative dementia, and a high percentage of cases show Alzheimer´s disease (AD) copathology. Patients with DLB and concomitant AD have increased risk of nursing home admission, shorter survival rates, and faster progression to dementia.

The aim of this doctoral thesis is to expand our knowledge about the interaction of these two brain proteinopathies by analyzing: demographic, clinical features, global cognition, regional brain atrophy, and AD cerebrospinal fluid (CSF) biomarkers of DLB patients from the European dementia with Lewy bodies consortium (E-DLB) cohort.

Our findings demonstrate that AD-related pathology is associated with posterior brain atrophy in patients with DLB, while amyloid-β related pathology is associated with atrophy in the medial temporal lobe. Further, DLB patients with amyloid-β related pathology present a faster cognitive decline, whereas tau-related pathology does not seem to be linked to cognitive worsening. Finally, we have found that DLB is an heterogeneous disease with endophenotypes that present distinctive demographic and clinical features, as well as different regional brain atrophy and AD CSF profiles.

In conclusion, the coexistence of AD pathology influences the neurodegenerative process, longitudinal cognitive decline, and heterogeneity in patients with DLB.

Resumen

La demencia con cuerpos de Lewy (DCL) es la segunda causa más frecuente de demencia neurodegenerativa, y un alto porcentaje de casos muestra copatología con la enfermedad de Alzheimer (EA). Los pacientes con DCL y EA concomitante tienen un mayor riesgo de ingreso en residencia, menor tasa de supervivencia y progresión más rápida a la demencia.

El objetivo de esta tesis doctoral es ampliar nuestro conocimiento sobre la interacción de estas dos proteinopatías cerebrales a través del análisis de: características demográficas y clínicas, cognición global, atrofia cerebral regional y biomarcadores de EA en líquido cefalorraquídeo (LCR) de pacientes con DCL de la cohorte del consorcio Europeo de la demencia con cuerpos de Lewy (E-DLB).

Nuestros hallazgos demuestran que la patología relacionada con la EA se asocia con atrofia cerebral posterior en pacientes con DCL, mientras que la patología relacionada con el amiloide-β se asocia con atrofia en el lóbulo temporal medial. Además, los pacientes con DCL que muestran patología relacionada con amiloide-β presentan un deterioro cognitivo más rápido, mientras que la patología relacionada con tau no parece estar ligada a empeoramiento cognitivo. Por último, hemos encontrado que la DCL es una enfermedad heterogénea con endofenotipos que presentan características demográficos y clínicas distintivas, así como diferentes perfiles de atrofia cerebral regional y de biomarcadores de EA en LCR.

En conclusión, la coexistencia de patología de la EA influye en el proceso neurodegenerativo, el deterioro cognitivo longitudinal y la heterogeneidad de los pacientes con DCL.

1. Introduction

Our brain is the most complex system known by us in the Universe, and is the mean we use to explore what surround us, helping us to unravel our most deep questions: why are we here? What are we? How did we come into existence?

In order to answer these questions, our brain has a wide range of tools. One of these amazing features is its ability to generate patterns. Pattern recognition enable us to make sense of the world by classifying and ordering the information we get from our senses, helping us to spot danger, make decisions, and secure the species survival (1). But the generation of a pattern, does not mean that it exists in reality. For example, ancient civilizations established constellations from patterns of grouped stars in the sky, usually to measure time. These patterns help them to decide when to sow, guide travels or held religious festivities. Yet, these brain imaginary arrangements are not associated in reality, and the stars in a constellation can be separated by light years. Therefore, pattern recognition is a very useful tool, but we must not forget that patterns are brain-made, and the reality could be far more complex than we first imagine.

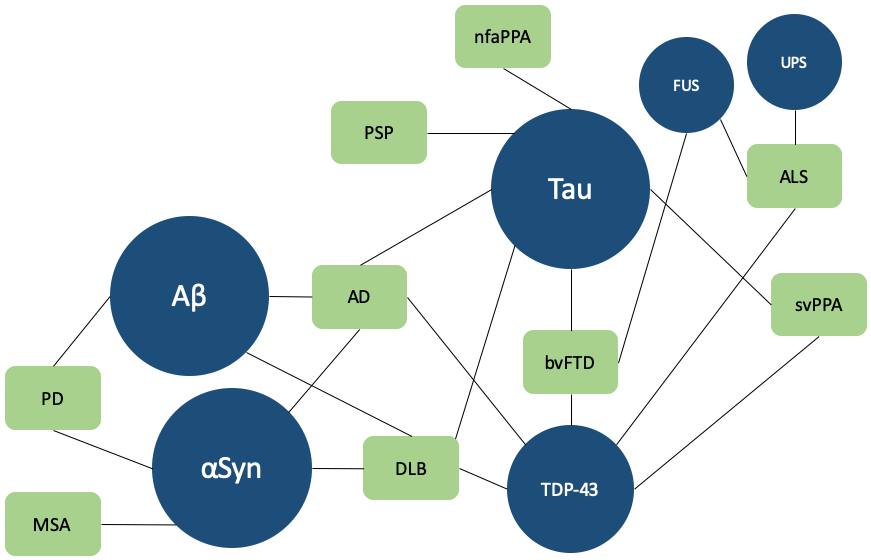


Figure 1. Scheme of neurodegenerative proteinopaties

Modified from Allegri, 2020; Golde, Borchelt, Giasson, & Lewis, 2013. Aβ: amyloid-β, AD: Alzheimer´s disease, ALS: amyotrophic lateral sclerosis, αSyn: alpha-synuclein, bvFTD: behavioural variant of frontotemporal dementia, DLB: dementia with Lewy bodies, FUS: fused-in sarcoma protein, MSA: multi system atrophy, PD: Parkinson´s disease, nfaPPA: nonfluent agrammatic primary progressive aphasia, svPPA: semantic variant of primary progressive aphasia, PSP: progressive supranuclear palsy, TDP-43: TAR DNA-binding protein 43, UPS: ubiquitin proteasome.

In medicine we often use patterns to classify and order diseases. This is the methodology we have used to categorize neurodegenerative diseases according to the accumulated proteins that we are able to identify with the current technology. But in reality, there are several proteins accumulated in the brain and probably some more that we have not identified yet.

So how do these proteins interact with each other? What is their role in disease progression? Can we treat one of them and hope to stop cognitive and functional decline?

This is the aim of this doctoral thesis: increase our knowledge about the interaction of the brain proteinopathies responsible for most cases of neurodegenerative dementia: DLB and AD.

In particular, this thesis is focused on the influence of AD-related pathology in the neurodegenerative process, longitudinal cognitive decline, and heterogeneity of DLB patients. Thus, we have analyzed the association between abnormal levels of AD CSF biomarkers and regional brain atrophy in DLB patients; we have studied the longitudinal cognitive performance of DLB patients with normal and abnormal levels of AD CSF biomarkers; and we have parsed the heterogeneity in DLB by using a multimodal subtyping method to identify subpopulations of patients with common demographic, clinical, magnetic resonance imaging (MRI) and AD CSF profiles.

The study of mixed dementias is relevant, especially for DLB patients with concomitant AD, who represent almost 70% of cases. These patients present worse health indicators with lower survival rates, increased risk of nursing home admission, and a faster progression to dementia. The reality is expected to be more complex than the patterns we have established, but we have to investigate and embrace this complexity if we are to find a cure for dementia.

1.1 Lewy body disease definition

Lewy body disease is a type of neurodegenerative disorder that consists in the deposit of intracytoplasmatic inclusions composed of α-synuclein and ubiquitine named Lewy bodies and Lewy neurites. This entity includes two syndromes: Parkinson´s disease (PD) and DLB (Figure 1) (4) This work will focus on DLB.

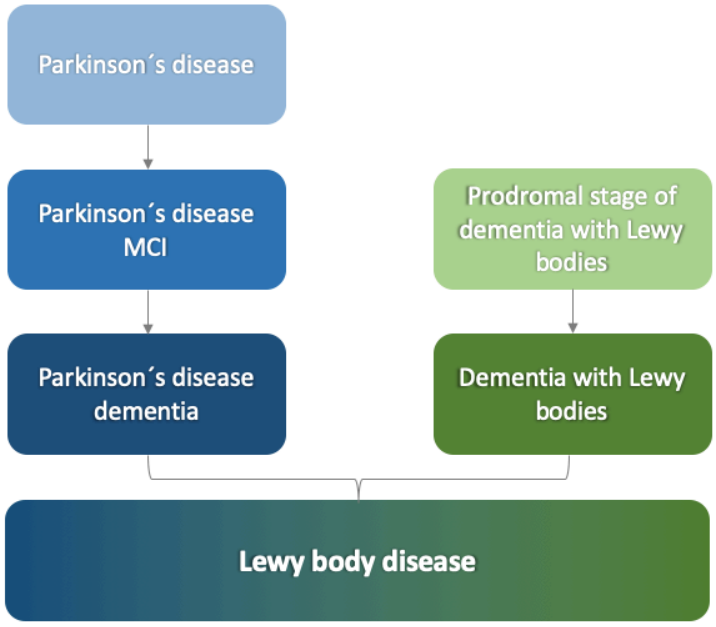


Figure 2. Lewy body dementia spectrum
MCI: mild cognitive impairment.

1.2 Epidemiology

DLB is the second most common cause of dementia after AD (5,6). Its prevalence remains unknown and depends on the setting, ranging from 0% to 30.5% of all dementia cases in population based studies (7) Recent studies have found a community prevalence of 4.2% to 4.6% of all dementia cases (8,9) Interestingly, this prevalence increases up to 7.5% in secondary care, probably due to a more accurate diagnosis (8) But when applying neuropathological diagnosis, Lewy body pathology is present in approximately 20% of postmortem brains (10) The difference between clinical and pathological diagnosis, indicates that DLB is an underdiagnosed disease.

With regard DLB incidence, the annual incidence rate is approximately 4% of new dementia diagnoses (7,8) and 0.5 to 1.6 per 1000 person-years in community-dwelling people over 65 years old (11).

In terms of gender distribution, traditionally DLB has been considered more common in men (12,13), but some prevalence studies have found female predominance (8) Therefore, gender

distribution in DLB presents mixed results, and future research is needed to determine if this is due to the absence of sex differences or methodological biases.

In relation to risk factors: family history of PD, history of depression, anxiety or stroke and being an *APOE* ε4 carrier increase the likelihood of DLB diagnosis, whereas caffeine use and history of cancer decrease its probability (13).

In comparison to AD, DLB harbors a higher mortality risk (14–16). Additionally, these patients have more functional impairment, more impact in their quality of life, increased healthcare costs, earlier nursing home admission and higher rates of hospital admission (17–22).

1.3 Diagnosis

In 1912, Fritz Jakob Heinrich Lewy first described the eosinophilic intracytoplasmatic inclusions named after him, as part of his neuropathological studies on *Paralysis agitans* (23) But it was not until 1961 when Okazaki et al associated the presence of cortical Lewy bodies with dementia in 2 autopsied cases (24) Then, in 1976 Kenji Kosaka made the first complete description of DLB in an autopsied case. Interestingly, this case was clinically diagnosed with atypical presenile AD with parkinsonism, and the autopsy showed cortical and brain stem Lewy bodies with concomitant AD pathology (25,26) During the 1980´s, reports from Japan, Europe, and USA lead the discussions about the proper terminology for this nosological entity; until 1995 when the term *dementia with Lewy bodies* was proposed during the first International Workshop on DLB held in New Castle. A year after, DLB diagnosis was operationalized through international consensus criteria. This collaborative effort started with the publication of the "Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop" (27). This guideline has been updated in 2005 (28) and more recently in 2017, when the last version of the consensus was published (29).

The current definition of DLB is: a syndrome characterized by cognitive impairment sufficient enough to impair the patient´s ability to perform activities of daily living, accompanied by parkinsonism, cognitive fluctuations, REM sleep behaviour disorder (RBD) and/or recurrent visual hallucinations (29).

DLB diagnosis relies mainly in clinical features that can be divided in three categories: cognitive, behavioral/psychiatric and physical symptoms (30).

From a cognitive standpoint, DLB patients tipically present attentional, executive function and visual processing deficits (31–33) Conversely, confrontation naming and episodic verbal memory usually are less affected (34,35) For DLB diagnosis, the cognitive impairment must be progressive and sufficient enough to cause alteration in the patient´s functionality, meaning a diagnosis of dementia is essential. Cognitive deficits in DLB patients may fluctuate, as well as level of alertness and attention. These fluctuations are a core feature of the disease, and are defined as "alternating episodes of normal or almost normal function with periods of impaired cognitive performance, inattention and excessive daytime drowsiness with transient confusion on waking" (27).

Regarding the behavioral and psychiatric symptoms, patients with DLB can have hallucinations, delusions, anxiety, depression, apathy and RBD (30). Recurrent visual hallucinations are another core feature, present in approximately 80% of patients with DLB (29) In early stages of the disease, hallucinations are the best predictor of DLB´s anatomopathological diagnosis, and sometimes they are the only initial sign of the disease in the mild dementia stage (36,37). DLB patients are able to report this symptom, which usually features people or animals (29) Other psychotic symptoms, like hallucinations in other modalities or systematized delusions, can ocurr in the course of the disease (28) RBD consists in the presence of involuntary movements during the atonic phase of the sleep. This symptom is strongly associated with underlying synucleinopathy, and is frequently present in autopsy-confirmed DLB cases, whereby it has been included as a core feature in the latest version of the diagnostic consensus criteria (38–40). Another sleep disturbance that has been added as a supportive clinical feature is hypersomnia (41) Other symptoms such as depression and anxiety are present in approximately 25% of DLB subjects, and retrospective research studies have found that history of depression and delirium are more frequent in patients with DLB than in AD (30).

The physical symptoms that DLB patients might present are: parkinsonism, hyposmia, constipation, autonomic dysfunction, sialorrea, among others. The manifestation of parkinsonism is one of the core features of the syndrome, can be its first sign in at least one quarter of patients (30) and is present in over 85% of patients (42) In DLB patients,

parkinsonism is defined as the presence of bradykinesia, rest tremor or rigidity (29) These symptoms must be present within the first year or at the same time of the diagnosis of dementia. This is known as the *"1-year rule"* and helps to differentiate DLB from PDD (29) Compared with PD patients, in DLB there is greater axial involvement, increased postural instability, gait disturbances and hypomimia; whereas rest tremor is less frequent (43) DLB diagnosis is less likely if parkinsonism is the only core feature and manifests at severe dementia stage. Postural instability, repeated falls, syncope, autonomic dysfunction, constipation, ortostatic hypotension, urinary incontinence, and hyposmia are considered supportive clinical features, and could be early findings more frequently present in DLB compared to AD (29,44,45).

Another supportive clinical characteristic is severe sensitivity to antipsychotics, previously listed in 2005 consensus criteria as a suggestive feature. It is characterized by acute onset of exacerbation of parkinsonian signs, and impaired level of alertness in patients treated with antipyschotics. Although up to 50% of DLB patients does not show this response to typical or atypical dopamine receptor D2 blocking agents, and their use as diagnostic strategy is not recommended, severe neuroleptic sensitivy strongly supports DLB diagnosis (28).

There is increased interest in the use and development of biomarkers for the diagnosis of DLB. This is reflected in 2017 DLB diagnostic criteria where biomarkers have gained greater importance. Here biomarkers are classified as indicative and supportive depending on their diagnostic specificity.

Indicative biomarkers are reduced dopamine active transporter (DAT) uptake in basal ganglia demonstrated by Single Photon Emission Computerized Tomography (SPECT) or Positron Emission Tomography (PET), reduced uptake on Iodine-123 metaiodobenzylguanidine myocardial (MIBG) scintigraphy, and rapid-eye-movement (REM) sleep without atonia confirmed by polysomnography (PSG) (29) Dopamine system neuroimaging using 123I-FP-CIT SPECT have shown a sensitivity and specificity higher than 80% in differentiating DLB from non-DLB dementias (46,47). The abnormal uptake evidenced in DLB patients has been associated with decreased nigral dopaminergic neuronal density but not to pathological deposition of α-synuclein, amyloid-β or tau (48) Another indicative biomarker with a sensitivity of 69% and specificity of 87% for distinguishing probable DLB from probable AD cases is MIBG. This noninvasive test helps to estimate

myocardial sympathetic degeneration found in DLB patients (49) Further, as common conditions in patients with dementia like periodic limb movements, confusional awakenings, hallucinatory-like behaviors or obstructive sleep apnea can be misdiagnosed as RBD, PSG confirmation of REM sleep without atonia is recommend since the likelihood of synucleinopathy is ≥ 90% in individuals dementia and RBD (39,50).

Supportive biomarkers for DLB diagnosis are relative preservation of medial temporal lobe structures on Computarized Tomography (CT) or MRI, generalized low uptake on SPECT/PET perfusion/metabolism scan with reduced occipital activity, posterior cingulate sign on Fluodeoxiglucose Positron Emission Tomography (FDG-PET) imaging, and prominent posterior slow-wave activity on electroencephalography (EEG) with periodic fluctuations in the pre-alpha/tetha range. These complementary tests can aid the diagnostic process but have less clear diagnostic specifity than the indicative biomarkers (29).

DLB diagnosis is based on the clinical features and biomarkers aforementioned, resulting in probable or possible DLB. For both diagnostic categories is essential the diagnosis of dementia. In addition, to diagnose a probable DLB case it is required the presence of at least two core features or one core feature plus at least one indicative biomarker. This diagnosis cannot be based only on biomarkers. Meanwhile, possible DLB diagnosis can be made when dementia plus only one core feature or one indicative biomarker is present. Table 1 summarizes 2017 DLB diagnostic criteria (29).

Table 1. Fourth consensus criteria for probable and possible dementia with Lewy bodies

Essential	Dementia	
	Clinical features	**Biomarkers**
Core	Recurrent visual hallucinations Fluctuating cognition REM sleep behavior disorder One or more spontaneous cardinal features of parkinsonism: bradykinesia, rest tremor or rigidity	Decreased dopamine transporter uptake in basal ganglia demonstrated by SPECT or PET Decreased uptake ^{123}iodine-MIBG myocardial scintigraphy Polysomnography confirmation of REM sleep behavior disorder
Supportive	Severe sensitivity to antipsychotic agents Postural instability Syncope or other transient episodes of unresponsiveness Systematized delusions Hallucinations in other modalities Repeated falls Severe autonomic dysfunction Hypersomnia Apathy, anxiety and depression Hyposmia	Relative preservation of medial temporal lobe structures on CT/MRI Generalized low uptake on SPECT/PET perfusion/metabolism scan with reduced occipital activity +/- the cingulate island sign on FDG-PET Prominent posterior slow-wave activity on EEG with periodic fluctuations in the pre-alpha/tetha range
PROBABLE DLB	2 or more core clinical features +/- an indicative biomarker 1 core clinical feature + one or more indicative biomarkers	
POSSIBLE DLB	1 core clinical feature One or more indicative biomarkers	

Modified from McKeith et al, 2017.

1.3 Neuropathological diagnosis

DLB neuropathological hallmarks are Lewy bodies and Lewy neurites, which are aggregates of α-synuclein in cell bodies and processes (51).

There are several clasiffications systems for DLB neuropathological staging, based on semiquantitative scoring and anatomical distribution of Lewy bodies and Lewy neurites (28,52–54) However, these systems have shown low inter-rater reliability and difficult case classification either by the lack of a category or because a case can be allocated to more than one category. Hence, Attems and colleagues have proposed a new classification system for Lewy pathology called Lewy pathology consensus criteria (LPC) (55). This consensus is

based on dichotomized scoring of Lewy bodies and Lewy neurites as present or absent in the following brain regions: olfactory bulb, dorsal motor nucleus of the vagal nerve, substancia nigra, amygdala, cingulate cortex, and medial-temporal, frontal and parietal cortices. The resulting categories are: olfactory only, amygdala predominant, brainstem predominant, limbic and neocortical Lewy pathology. These categories are shown in Table 2. This system permits the classification of all DLB cases and has demonstrated good inter-rater reliability.

Table 2. Lewy pathology consensus criteria proposed by Attems et al, Acta Neuropathologica 2021

Category of Lewy pathology	Olfactory bulb	Amygdala	Dorsal motor nucleus of the vagal nerve or substancia nigra	Medial temporal lobe or cingulate cortex	Frontal or parietal cortex
Olfactory only	+	-	-	-	-
Amygdala predominant	+/-	+	-	-	-
Brainstem predominant	+/-	+/-	+	-	-
Limbic	+/-	+/-	+/-	+	-
Neocortical	+/-	+/-	+/-	+/-	+

Yet, the LPC does not take into account concomitant AD pathology. This is addressed by the updated version of the pathologic assessment and diagnostic criteria suggested by McKeith et al in 2017. Here the authors present the likelihood that the pathological finding correspond to a probable DLB case considering both Lewy body pathology and AD neuropathological change according to National Institute on Aging-Alzheimer´s Association (NIA-AA) criteria as shown in Table 3 (29).

Table 3. Probability of DLB neuropathological diagnosis with respect Lewy body and AD pathology

AD pathologica change / Lewy-related pathology	NIA-AA none/low (Braak stage 0-II)	NIA-AA intermediate (Braak stage III-IV)	NIA-AA high (Braak stage 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

1.4 Genetics

DLB is mostly considered a sporadic disease because genetic causes are infrequent (56–58) However, genome-wide association studies (GWAS) have found that Apolipoprotein E (*APOE)*, Synuclein Alpha (*SNCA)* and Glucosylceramidase Beta (*GBA)* genes are involved in DLB (59,60).

APOE gene encodes apolipoprotein E, a protein with 3 isoforms (ε2, ε3 and ε4), implicated in lipid transport and expressed primarily in microglia and astrocytes. *APOE* ε4 is associated with amyloid-β accumulation and is a well-known genetic risk factor for AD (61). *APOE* ε4 is also a risk factor for DLB (62,63) found in both pure and mixed DLB cases with concomitant AD pathology, suggesting mechanisms unrelated to the amyloid cascade (63,64) In addition, *APOE* ε4 is a strong predictor of faster cognitive decline in DLB (65) and has been independently associated with greater severity of Lewy body pathology (66,67).

SNCA gene encodes α-synuclein, a presynaptic protein that regulates membrane fusion and synaptic transmission (68) Mutations and locus multiplications in *SNCA* gene are rare causes of DLB. Additionally, *SNCA* modulates disease risk in DLB and PD (69).

GBA gene encodes a lysosomal enzyme called β-glucocerebrosidase (Gcase). Heterozygous mutatios in *GBA* are a risk factor for DLB (70–72), and their carriers can present earlier disease onset (73,74). Furthermore, increased frequency of *GBA* mutations have been found in DLB patients without concomitant AD pathology, which could be associated with a more "pure" DLB phenotype (64,75).

In addition to *APOE*, *SNCA,* and *GBA* genes, mutations in genes that cause Mendelian forms of dementia can be present in a small percentage of DLB patients. Thus, *APP*, *PSEN1*, *PARK2* mutations have been identified in DLB cases, and there are reports of rare variants in *GRN* and novel variants in *MAPT* that need futher investigation (69).

1.5 Biomarkers of Lewy body related pathology

Although direct biomarker evidence of Lewy body pathology is not currently available, efforts are being made to detect in vivo one of its hallmarks: misfolded α-synuclein in biofluids, neuroimaging and tissues.

CSF and blood α-synuclein levels have been investigated using mainly immunoassays (76). Some studies have found abnormal CSF levels of α-synuclein in DLB (77–79) which could help to distinguish it from AD (80,81) Nevertheless, the inconsistency of the results, the wide variation in analytic performance and concentration of α-synuclein in biofluids below the detecting range of standard techniques, hampers the use of conventional immunoassays for diagnostic purposes (82–85) A novel approach called Real-Time Quaking-Induced Conversion (RT-QuIC) could help to overcome these limitations. RT-QuIC amplifies in vitro the pathogenic protein seed into amyloid fibrils that bind to an amyloid-sensitive dye called thioflavin T (ThT) producing an enhanced fluorescence (86) "Real time" ThT fluorence readings of the pathogenic protein seed have shorter lag-phases than unseeded reactions (86) This new technique have been used in CSF of patients with Lewy body disease for the detection of α-synuclein showing high sensitivy and specificity (87,88).

Another challenge for the assessment of α-synuclein in biofluids is its large expression outside the central nervous system. In blood, the major source of α-synuclein are red blood cells (>99%) (89) Therefore, red blood cells could be used as biomarkers for dementia (90). Yet, they can also be a source of contamination of serum and plasma resulting in a false increase of α-synuclein levels. Despite these constraints, low concentrations of α-synuclein have been found in serum and red blood cells of DLB patients (91,92).

In addition to biofluids, PET imaging is being studied for the detection of Lewy body pathology in vivo. Several compounds have been developed for the identification of α-synuclein, of which only two have shown high affinity to α-synuclein fibrils: [^{11}C]MODAG-

001 and [^{125}I]TZ6184 (93). However, [^{11}C]MODAG-001 has not been able to detect aggregated α-synuclein in human brain tissue from DLB patients (94); and the selectivity profile of [^{125}I]TZ6184 has not been reported yet (93).

The development of α-synuclein PET tracer remains elusive for numerous reasons: the low concentration of α-synuclein aggregates in the brain (approximately 10 to 5-fold lower than that of amyloid-β or tau), the co-localization of α-synuclein aggregates with amyloid-β and tau fibrils which hampers selectivity, the need to cross both the cell membrane and the blood-brain-barrier given that α-synuclein inclusions are mainly intracellular, and the lack of reliable and reproducible assays (93,95) Nevertheless, several research groups around the world are working to develop reliable α-synuclein PET radioligands.

Other potential sources for α-synuclein detection are sample tissues. Skin, neural structures of the submandibular gland and gastrointestinal mucosa biopsies of PD patients have shown increased deposition of α-synuclein whem compared to controls (96,97) Similarly, pathological α-synuclein deposition has been found in sample tissues from the submandibular gland and skin of patients with DLB (98,99) Yet, more studies are needed for the validation of these biomarkers in clinical settings.

1.6 Alzheimer´s disease copathology

Amyloid-β and tau pathologies are often found in DLB neuropathological studies and range from moderate to a severe degree in a high percentage of cases (4,64,100–102).

Fortunately, concomitant AD pathology can be studied in vivo thanks to the availability of CSF and PET biomarkers. Thus, CSF studies have shown that approximately 40% of patients with DLB present abnormal CSF levels of amyloid-β and tau (103–105) whereas PET neuroimaging results have demonstrated an increased amyloid-β load in approximately 50% of DLB patients (106,107). These results have been replicated in autopsy-confirmed studies showing that DLB patients with mixed AD pathology present decreased levels of amyloid-β42 (Aβ42) and increased levels of total tau (T-tau) in CSF (108,109).

The link between α-synuclein, amyloid-β and tau is not fully understood. Yet, there are some evidence of the interactions between these proteinopathies. On one hand, it has been found

a positive correlation between amyloid-β load and the amount of α-synuclein deposits, which seems to depend on the number of primitive and mature amyloid-β plaques in the cortex (110) With regards to tau, frequent colocalization with α-synuclein has been observed in specific neuronal populations (110–112) The underlying mechanisms are unknown, but results from in vitro studies suggest that tau overexpression influences α-synuclein pathological aggregation, augmenting the number of aggregates, the levels of insoluble α-synuclein and its cytotoxicity (113).

The study of DLB with concomitant AD pathology is important because these mixed cases are frequent and present worse health indicators having increased risk of nursing home admission, shorter survival rates, and a faster progression to dementia (64,105,114–116) Therefore, the aim of this thesis is to analyse the influence of AD-related pathology in the regional brain atrophy, longitudinal cognitive decline and heterogeneity of patients with DLB.

2. Hypothesis

2.1 Principal hypothesis:

- DLB is influenced by AD-related pathology.

2.2 Specific hypotheses:

- CSF levels of AD biomarkers are associated with regional brain atrophy in DLB patients.
- CSF levels of AD biomarkers predict longitudinal cognitive decline in DLB patients.
- DLB patients are an heterogeneous group conformed by endophenotypes with distinct demographic, clinical, regional brain atrophy, and AD CSF biomarkers profiles.

3. Objectives

3.1 Principal objective

- To analyze the influence of AD-related pathology in DLB.

3.2 Specific objectives

- To study the association between CSF levels of AD biomarkers and regional brain atrophy in DLB patients.
- To study longitudinal cognitive performance of DLB patients with normal and pathological levels of AD CSF biomarkers.
- To parse the heterogeneity in DLB by using multimodal subtyping method to identify endophenotypes of patients with common demographic, clinical, regional brain atrophy, and AD CSF biomarkers profiles.

4. Materials and Methods

The methodology used to determine the combined effect of regional brain atrophy and AD CSF biomarkers, and association between longitudinal decline and AD CSF biormarkers, has been published by Abdelnour et al (117,118). In addition, the methods applied to analyze DLB heterogeneity has been submitted for publication to the Alzheimer´s Research & Therapy journal.

Here we present the compiled information for the materials and methods used in these manuscripts.

4.1 Participants population

Participants were selected from the E-DLB cohort (119). The E-DLB consortium archives data from more than 40 centers across Europe, including patients with DLB, PDD, and AD.

We used the following inclusion criteria: 1) diagnosis of probable DLB; and 2) availability of AD CSF biomarkers data. Additionally, for the analysis of regional brain atrophy we selected patients with MRI data; and for the study of longitudinal cognitive decline we selected subjects with MMSE scores available at baseline and at 1 or 2 years of follow up.

Patients from eight centers satisfied these criteria: Memory Clinic, Karolinska University Hospital, Huddinge; Clinical Memory Research Unit, Department of Clinical Sciences, Lund University; Neuropsychology Unit and Geriatric Day Hospital, Strasbourg Resourse and Research Memory Center, University Hospital of Strasbourg; Center for Age-Related Medicina, Stavanger University Hospital; Alzheimer Center Amsterdam, Amsterdam UMC; Ace Alzheimer Center Barcelona; Department of Neurology, Ljubljana University Medical Center; and Neurology Unit, Department of Clinical and Experimental Sicences, University of Brescia. In table 4, we present the number of patients included by center considering the inclusion criteria for each substudy.

Table 4. Number of patients included by center and study

Substudy	E-DLB Center	Number of DLB patients
Regional brain atrophy	Strasbourg	39
	Stockholm	24
	Brescia	6
	Stavanger	6
	Ljubljana	6
	Barcelona	5
Longitudinal cognitive decline	Mälmo	50
	Strasbourg	32
	Stockholm	11
	Stavanger	7
Heterogeneity	Amsterdam	38
	Strasboug	38
	Stockholm	17
	Brescia	6
	Barcelona	5

4.2 Diagnostic and clinical examination

The E-DLB cohort was assembled retrospectively, thus DLB diagnosis was made according to McKeith 2005 criteria (28). Diagnosis was made by the treating physician, a group of at least two expert clinicians, or a multidisciplinary team at a consensus diagnostic meeting on the basis of all available clinical and diagnostic test data as previously reported (119,120).

Clinicians interviewed both patients and caregivers, recorded demographic information as well as medical and drug history. We excluded patients with acute delirium, terminal illness, stroke, psychotic or bipolar disorder, craniocerebral trauma, or a major neurological illness other than dementia. All centers recorded whether patients fulfilled criteria for parkinsonism, visual hallucinations (VH), cognitive fluctuations, and a clinical history of probable RBD. These core diagnostic features were recorded as present or absent. Neuropsychological evaluation and complementary tests to rule out secondary causes of dementia (routine blood tests and brain imaging) were performed. The Mini-Mental State Examination (MMSE) was scored as a measure of global cognition (121) at baseline and annually for up to two years.

4.3 Ethics

Local ethics committees at the individual centers approved the study. The patients gave their written consent to use the anonymised results of their clinical, instrumental and laboratory investigations for research purposes.

4.4 CSF procedures

CSF was obtained at all centers with the following procedures: (1) lumbar puncture at the L3-4 or L4-5 interspace; (2) collection in polypropylene tubes and centrifuged for 10 minutes at 4°C; and (3) storage in aliquots of 0.5 mL at -80°C or -70°C until further analysis. Further details are summarized in Annex I: Supplementary Table 1. CSF analyses were performed locally according to standard routines. T-tau and phosphorylated tau at threonine 181 (p-tau) were analyzed with INNOTEST enzyme-linked immunosorbent assays (ELISA). Aβ42 was analyzed with INNOTEST ELISA in all center but Stavanger, that used ELISA kits from Biosource Europe S.A. CSF values were dichotomized as normal or pathological based on well-established center-specific cut-off values for each biomarker.

We used two definitions of an AD CSF profile, due to the timing of the analysis with respect to the publication of the NIA-AA research framework of the amyloid-β deposition, pathologic tau, and neurodegeneration [AT(N)] classification (122).

The first definition was established for the analysis of the association between AD CSF biomarkers and longitudinal cognitive decline performed in 2016. Here, we defined an AD CSF profile as abnormal CSF levels of Aβ42 combined with abnormal CSF levels of T-tau or p-tau (123). Based on this profile, DLB patients were divided in an AD CSF profile pathological group and an AD CSF profile normal group.

This definition was uptaded for the study of DLB heterogeneity conducted in 2020. In this analysis patients were divided in three groups: 1) AD pathological change= abnormal CSF levels of Aβ42 alone, 2) AD pathology= abnormal CSF levels of Aβ42 combined with abnormal CSF levels of p-tau, and 3) amyloid-independent tau-pathology= abnormal CSF levels of p-tau combined with normal CSF levels of Aβ42.

4.5 MRI analysis

Various MRI scanners and protocols were used as detailed in Annex I: Supplementary Table 2. Due to variability in MRI scanners and protocols, we favored visual rating scales by an experienced neuroradiologist (L.C.) rather than application of automated methods for regional brain atrophy. The neuroradiologist was blind to any clinical information including diagnosis. Regional atrophy was assessed with three visual rating scales using T1-weighted images as detailed elsewhere (124). Briefly, atrophy in the medial temporal lobe was assessed with the MTA scale (125); atrophy in the posterior cortex was assessed with the PA scale (126); and atrophy in the frontal lobe was assessed with the GCA-F scale (127). In the three visual rating scales, higher scores indicate an increasing degree of atrophy. MTA analysis was based on coronal reconstructions, GCA-F on axial reconstructions, and PA on reconstructions from all three planes. The neuroradiologist who evaluated the images (L.C.) has previously demonstrated excellent intra-rater reliability in 120 random cases: weighted kappa values of 0.94 and 0.89 for MTA in left and right hemispheres, respectively, 0.88 for posterior atrophy (PA), and 0.83 for global cortical atrophy scale–frontal subscale (GCA-F) (124).

For the analysis of heteroteneity in DLB, we included the assessment of white matter hyperintensities (WMHs) on axial FLAIR images, as a marker of cerebrovascular disease, using the Fazekas scale (128). This evaluation was perfomed by the same neurorradiologist (L.C.). Briefly, the Fazekas scale grades WMHs as 0 (i.e. absence of WMHs), 1 (i.e. punctate WMHs), 2 (i.e. early confluent WMHs), and 3 (i.e. WMHs in large confluent areas). Fazekas scores were classified into low (Fazekas scores 0 or 1) and high (Fazekas scores 2 or 3) WMH burden, as in previous studies (129,130).

4.6 Statistical analysis

The statistical analyses were done using R (www.R-project.org) version 3.2.4, and IBM SPSS versions 20 and 26. Results are shown as mean ± SD for normally distributed continuous variables, median [range] for non-normally distributed continuous variables, and number and percentage for categorical variables. A p-value ≤ 0.05 was deemed statistically significant.

Below we describe in detail the specific statistical methods applied to test each hypothesis.

4.6.1 Statistical analysis of the combined effect of AD CSF biomarkers on regional brain atrophy

Here our aim was to investigate the combined effect of Aβ42, T-tau and p-tau (predictors) on regional brain atrophy as measured with visual rating scales (outcome variables). All these measures are dichotomous (0 normal, 1 abnormal). We also wanted to model the effects of age, sex, education and disease duration to investigate their possible added effect to the association between CSF biomarkers and regional brain atrophy. Age, education and disease duration are continuous variables while sex is dichotomous (0 males 1 females). Further, our interest was to investigate the predictive power of all these variables in combination as predictors of regional brain atrophy, rather than investigating their partial effects. Random forest (classification) (131) was thus chosen given our aim, the nature of the variables, the number of predictors and the sample size. Random forest is an ensemble method in machine learning that involves growing of multiple decision trees via bootstrap aggregation (bagging). Each tree predicts a classification independently and votes for the corresponding class. The best model for each outcome variable is chosen from the majority of votes (132). Importantly, random forest investigates combined effects (the predictors do not compete with each other but "cooperate" in the prediction of the outcome) (132). In contrast to other predictive methods such as multiple linear or logistic regression that investigate partial effects (competition among predictors in the prediction of the outcome). Combined effects are closer to what we hypothesized in this study, i.e., amyloid-β (CSF Aβ42) and tau-related (CSF p-tau) pathologies have a synergistic deleterious effect on brain integrity. When CSF Aβ42 and CSF p-tau as predictors show a contribution to the prediction of brain atrophy, we might conclude that both pathologies have a combined effect on brain integrity, which may reflect their synergy at the pathological level (i.e. the "cooperation" between Aβ42 and CSF p-tau contributes to the prediction of brain atrophy). Further, random forest performs very similarly to other machine learning algorithms (132) but it was preferred in our current study due to the nature of our variables. We performed three random forest models: one for each atrophy scale (MTA, PA, and GCA-F) as the outcome variable. The random forest models were comprised of 5000 trees, providing an accurate estimation

of the variables importance without introducing too much noise in the models due to the addition of redundant trees. Each of the trees was trained on randomly picked 70% of the data and subsequently tested on the unseen 30% of the data. Classification models (normal *vs.* abnormal) (133) were conducted, accounting for the fact that the outcome variable may present with an unbalanced amount of cases in its two levels (e.g. normal MTA n = 53, abnormal MTA n = 34). The classification error is reported as a measure of goodness of the model (out-of-the-bag estimated error rate, OOB-EER) (131). When outcome variables are dichotomous, as it is our case, the error by chance is 50%. Therefore, a classification error below 50% is better than chance, with values closest to 0% denoting better classification performance, hence good reliability of the model. We also report the importance (*Imp*) of the predictors as a measure of their contribution towards the prediction of the outcome variable (regional brain atrophy). Higher *Imp* values denote stronger contribution to the prediction. The random forest results were further complemented with the Pearson correlation coefficient to easily represent the magnitude and direction of the association between variables (bivariate association). P-values of Pearson correlation are reported for completeness of information.

4.6.2 Statistical analysis for association of AD CSF biomarkers and longitudinal cognitive decline

Comparisons of baseline clinical and demographic data in the CSF profile groups were performed using parametric Student t-test and nonparametric Mann-Whitney U test as appropriate. Linear mixed effect (LME) models were used to determine whether the rate of cognitive decline measured by MMSE during the 2 year follow up was predicted by the AD CSF profile, followed by the specific CSF measures, i.e. Aβ42, t-tau, and p-tau, all with pathological or normal values. The impact on decline is represented by the interaction term between factor and time (year of follow up), adjusted for age, gender and education. The LME analysis included also the baseline value, which was therefore not adjusted for as a co-factor. There is considerable individual variation in both level and decline of MMSE and therefore LME models with both random intercept and random slope were used. Thus, the statistical model underlying the LME analyses captures both these kinds of individual variation.

4.6.3 Statistical analysis of heterogeneity in DLB

To parse heterogeneity and identify different subgroups of patients we conducted 2 steps.

In the first step, we aimed to identify the latent dimensions/components in the data that determine DLB heterogeneity. Since our data included both continuous and categorical variables, we used a multivariate method for data analysis called factorial analysis of mixed data (FAMD) (134). The main strength of FAMD is that it accommodates both quantitative and qualitative data simultaneously. FAMD works as a principal component analysis for quantitative data and as a multiple correspondence analysis for qualitative data (134). In our FAMD model, age, years of education, MMSE scores, and disease duration were included as continuous variables; and sex (male vs. female), CSF Aβ42, p-tau and t-tau levels, MTA, PA, and GCA-F scales (normal vs. abnormal); and parkinsonism, visual hallucinations, cognitive fluctuations, and probable RBD (absent vs. present) were included as categorical variables. Fazekas scores (low vs. high WMH burden) were not included in the FAMD model and subsequent cluster analysis due to missing data, but they were used to characterize the resulting subgroups, post-hoc.

In the second step, we aimed to classify patients into subgroups using a cluster analysis based on the dimensions provided by the FAMD model. We applied an agglomerative hierarchical clustering algorithm with the Ward's linkage method (135). This clustering method starts by assigning every DLB patient to one cluster and sequentially combines pairs of clusters at each step while minimizing the sum of square errors from the cluster mean. The algorithm continues merging DLB patients into clusters until all the patients form a single group. We identified the optimal number of clusters by using the Calinski-Harabasz criterion (136) and by visual inspection of the dendrogram from the agglomerative hierarchical clustering.

We characterized the resulting subgroups using one-way ANOVA for continuous variables, with t-test for post-hoc pair-wise analysis, using the Hochberg's correction for multiple testing (137). Chi-square test was used for categorical data.

5. Results

To facilitate the interpretation of the results, we present them according to the specific objectives described in Chapter II.

5.1 Study of the association between AD CSF biomarkers and regional brain atrophy

These results have been published by Abdelnour and colleagues in 2020 (117).

5.1.1 Sample features

In this analysis we selected 86 probable DLB patients who had CSF and MRI data available. Clinical and demographic features of the sample are reported in Table 5.

Table 5. Clinical and demographic features of the sample

Features	Mean (SD)	Range
Age at diagnosis	69.36 (8.85)	49-88
Sex: Male N (%)	49 (56.98)	
Years of education	11.24 (4.08)	5-22
Disease duration (years)	4.04 (3.10)	0.5-14
MMSE	24.85 (3.72)	15-30
Parkinsonism (%)	82.6 (N= 71)	
Visual hallucinations (%)	58.1 (N= 50)	
Fluctuating cognition (%)	75.6 (N=65)	
N: number. MMSE: Minimental State Examination		

Of the 86 patients, the number of patients with pathological CSF values was 28 (32.56%) for Aβ42, 17 (19.77%) for T-tau and 24 (27.91%) for p-tau. The number of patients with abnormal scores in the visual rating scales was: MTA: 33 (38.37%), GCA-F: 34 (39.53%) and PA: 45 (52.33 %). Figure 3 shows 3 examples of different combinations for CSF Aβ42, CSF p-Tau and the visual rating scales.

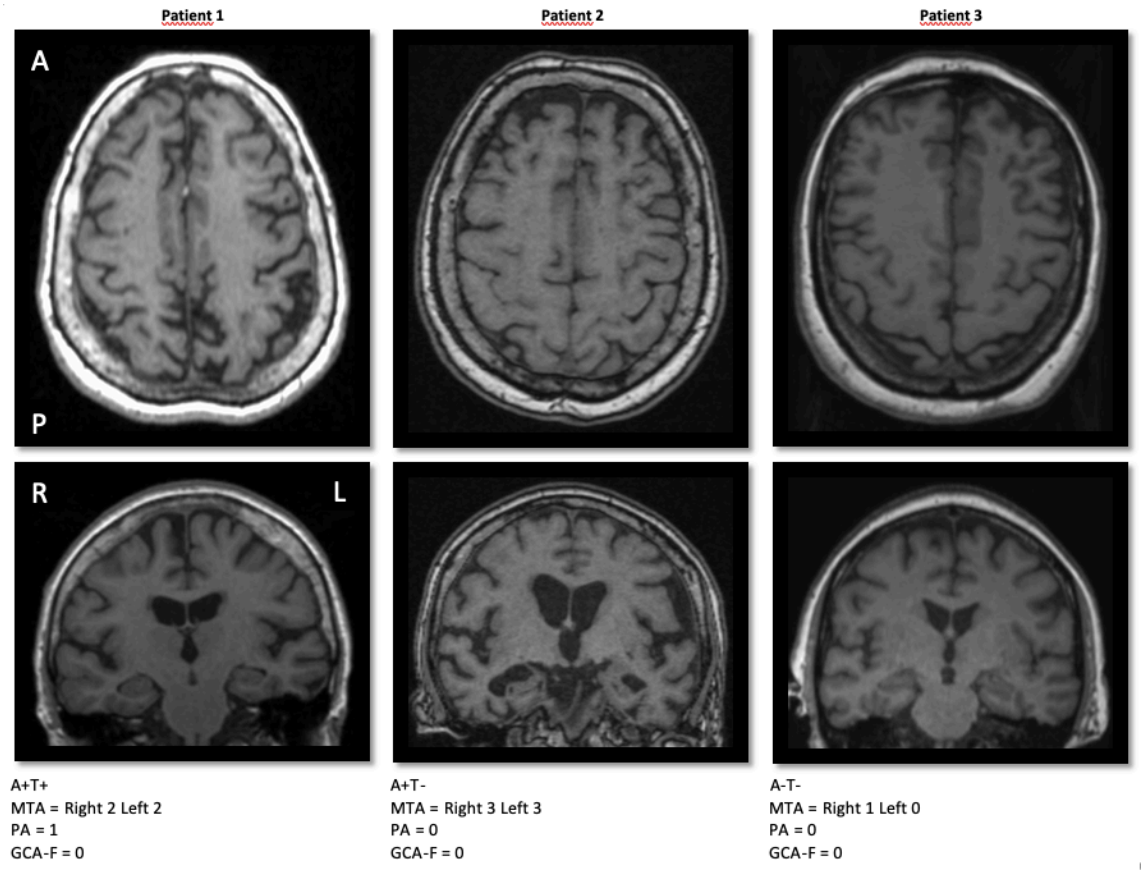


Figure 3. Normal and pathological CSF values of Aβ42 and p-Tau combined with visual rating scales

CSF levels of Aβ42 and p-Tau were dichotomized according the cut-offs of each center into normal or pathological values. MTA, PA and GCA-F visual rating scales were used to measure regional atrophy based on T1-weigthed images. A+ : pathological CSF Aβ42; A- : normal CSF Aβ42; T +: pathological CSF p-Tau; T- : normal CSF p-Tau; MTA: medial temporal atrophy scale; PA: posterior atrophy scale; GCA-F: global cortical atrophy scale – frontal subscale; A: anterior part of the brain; P: posterior part of the brain; R: right; L: left.

Twenty six out of the total sample of 86 subjects had available DAT SPECT, 25 (96.15%) of which were abnormal.

Additionally, the interval between MRI and CSF collection ranged from 0 to 3 months in the majority of the cases (73 out 86, which corresponds to 84.88%). In the rest of the patients (13 subjects) the interval ranged from 3 to 12 moths (15.12%).

5.1.2 Association between AD CSF biomarkers and visual rating scores measured with MRI

Table 6 present the distribution of abnormal scores in the visual rating scales in relation to normal or pathological CSF Aβ42, T-tau and p-tau.

Table 6. Distribution of abnormal visual rating scores between normal and pathological AD CSF biomarkers groups

	CSF Aβ42		**CSF T-tau**		**CSF p-tau**	
Visual rating scales	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Abnormal MTA N (%)	18 (54.55)	15 (45.45)	28 (84.85)	5 (15.15)	26 (78.79)	7 (21.21)
Age (mean and SD)	69.78 (8.37)	73.93 (6.68)	70.86 (8.01)	76.20 (5.07)	70.27 (8.13)	76.86 (3.34)
Sex (Male N and %)	11 (61.11)	6 (40)	18 (64.29)	2 (40)	17 (65.38)	3 (42.86)
Disease duration (mean and SD)	3.64 (2.91)	2.43 (2.35)	3.11 (2.80)	3.00 (2.35)	2.87 (2.54)	3.93 (3.32)
Abnormal PA N (%)	25 (55.56)	20 (44.44)	33 (73.33)	12 (26.67)	29 (64.44)	16 (35.56)
Age (mean and SD)	67.24 (9.40)	75.70 (6.78)	70.24 (8.22)	73.08 (11.88)	69.17 (8.07)	74.31 (10.62)
Sex (Male N and %)	15 (60)	12 (60)	20 (60.61)	7 (58.33)	18 (62.07)	9 (56.25)
Disease duration (mean and SD)	3.98 (2.69)	2.48 (1.57)	3.26 (2.24)	3.46 (2.78)	2.91 (1.91)	4.03 (2.96)
Abnormal GCA-F N (%)	19 (55.88)	15 (44.12)	27 (79.41)	7 (20.59)	20 (58.82)	14 (41.18)
Age (mean and SD)	70.68 (9)	75.93 (7.06)	71.30 (8.57)	79.57 (6.05)	70.95 (8.57)	75.93 (8.36)
Sex (Male N and %)	14 (73.68)	11 (73.33)	20 (74.07)	5 (71.43)	15 (75)	10 (71.43)
Disease duration (mean and SD)	4.61 (3.08)	2.77 (2.15)	3.63 (2.74)	4.43 (3.31)	3.18 (2.20)	4.68 (3.44)

N: number. CSF: cerebrospinal fluid. Aβ42: Amyloid-β_{42}. T tau: Total tau. P tau: phosphorylated tau at threonine 181. MTA: medial temporal lobe atrophy. PA: posterior atrophy. GCA-F: global cortical atrophy scale-frontal subscale.

Classification performance in the three random forest models was better than chance: MTA, OOB-EER = 32.56%; PA, OOB-EER = 44.83%, GCA-F, OOB-EER = 24.14% (Table 7).

The classification error for normal MTA was 24.52% and for abnormal MTA it was 45.45%. The classification error for normal PA was 46.34% and for abnormal PA it was 43.48%. The classification error for normal GCA-F scores was 19.23% while it was 31.43% for patients with abnormal values. Table 7 shows that the best predictors of MTA were disease duration, CSF Aβ42 and age, ordered by importance. We found a combined effect of CSF Aβ42 and CSF p-tau on PA. Age, education and disease duration also contributed to the prediction of PA. Finally, the best predictors of GCA-F were sex, education and age. AD CSF biomarkers did not contribute to the prediction of GCA-F. The same pattern of results was observed when adding the center as a predictor in the models (data not shown), thus suggesting that variability across-centers does not seem to affect our findings.

Pearson correlation coefficients show that abnormal scores in MTA were related to abnormal CSF Aβ42 levels, whereas abnormal values of PA were associated with both abnormal CSF Aβ42 and p-tau levels. Regarding the effect of age, sex, education and disease duration, abnormal scores in MTA were related to shorter disease duration and older age. Abnormal scores in PA were related to older age, less education and shorter disease duration. Abnormal scores in GCA-F were related to less education, male sex and older age (Table 7). Figure 4 shows the correlation matrix between visual ratings and CSF biomarkers, as well as among all predictors in our random forest models (131).

Table 7. Association between AD CSF biomarkers and visual rating scales (random forest models)

Visual rating scales	Variables contribution	Pearson correlation	P value
MTA	Overall model: OOB-EER = 32.56% - Classification error normal MTA = 24.53% - Classification error abnormal MTA = 45.45% Predictors retained in the model: Disease duration, *Imp*= 63.53 CSF Aβ42, *Imp*= 41.71 Age, *Imp*= 36.30	 -0.244 0.217 0.207	 0.024 0.045 0.056
PA	Overall model: OOB-EER = 44.83% - Classification error normal PA = 46.34% - Classification error abnormal PA = 43.48% Predictors retained in the model: Age, *Imp*= 24.27 Education, *Imp*= 8.76 CSF p-tau, *Imp*= 8.23 CSF Aβ42, *Imp*= 8.615 Disease duration, *Imp*= 7.02	 0.195 -0.166 0.179 0.266 -0.248	 0.072 0.126 0.100 0.013 0.021
GCA-F	Overall model: OOB-EER = 24.14% - Classification error normal GCA-F = 19.23% - Classification error abnormal GCA-F = 31.43% Predictors retained in the model: Sex, *Imp*= 54.20 Education, *Imp*= 52.18 Age, *Imp*= 46.23	 0.270 -0.231 0.334	 0.012 0.033 0.002

N: number. CSF: cerebrospinal fluid. Aβ42: Amyloid-β42. T-tau: Total tau. p-tau: phosphorylated tau at threonine 181. MTA: medial temporal lobe atrophy. PA: posterior atrophy. GCA-F: global cortical atrophy scale-frontal subscale. OOB-EER: out-of-the-bag estimated error rate (below 50% denotes good classification performance). *Imp*: importance (the contribution of a given variable in the random forest, with higher values indicating stronger contribution to the prediction). Pearson correlation indicates the direction of the association.

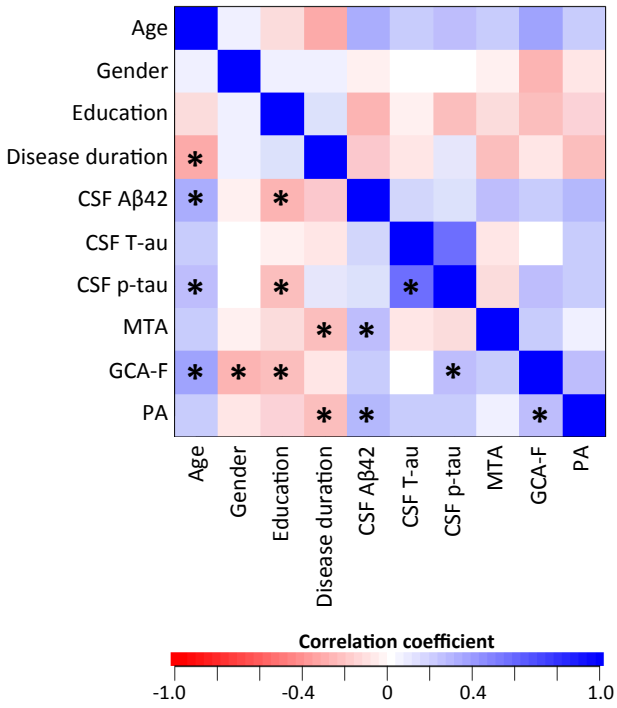


Figure 4. Correlation matrix between visual ratings, CSF biomarkers, and predictors in the random forest models

Asterisk symbols (*) denote p-values <0.05. MTA: medial temporal atrophy scale; PA: posterior atrophy scale; GCA-F: global cortical atrophy scale – frontal subscale.

5.2 Association between longitudinal cognitive decline and AD CSF biomarkers

These results have been published by Abdelnour and colleagues in 2016 (118).

5.2.1 Sample features

We selected 100 probable DLB patients who had MMSE scores at baseline and one year follow up, and 76 who had MMSE score at two years of follow up. Of the 100 subjects, 32% showed an AD CSF profile, 69% had a pathological value for Aβ42, 31.6% for T-tau (2 missing), and 26.9% for p-tau (7 missing). Baseline clinical and demographic variables showed no statistically significant differences between groups by AD CSF profile (Table 8).

DaTSCAN was performed in 38 DLB patients, 24 (63,16%) of whom had an abnormal updake.

Table 8. Demographics of DLB patients by AD CSF profile at baseline

Variable	AD CSF profile		
	Pathological (n=32)	Normal (n=68)	*P* Value
Age at baseline Mean±SD	74.22 ± 7.95	71.93 ± 7.79	0.176
Gender Male Female	 16 (26.2%) 16 (41.0%)	 45 (73.8%) 23 (59.0%)	0.122
Years of Education† Mean±SD	8.88 ± 3.34	10.63 ± 4.16	0.043
Disease duration§ Mean±SD	2.79 ± 1.94	3.01 ± 2.58	0.678
MMSE Median [IQR]	21.09 Range 5-30	22.68 Range 6-30	0.200
Numbers represent mean and SD, if not otherwise stated. Missing data: †Education 9 patients; § Duration 1 patient.			

5.2.2 CSF profile and cognitive decline

The overall rate of decline was 1.9 points per year. The LME analyses showed that the group with AD CSF profile was significantly associated with a more rapid decline, with 2.2 points per year (SE 1.1) higher annual decline in the AD CSF pathological group than the AD CSF normal group (p=0.04) (Figure 5) Male gender and higher level of education were both associated with more rapid decline (p<0.05).

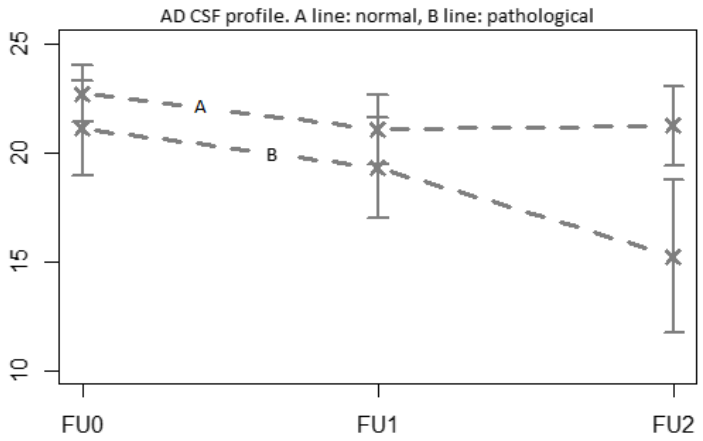


Figure 5. Change in MMSE score from baseline (FU0) to one (FU1) and two (FU2) years follow-up in those with (n=32) and without (n=68) a CSF AD profile
The difference was statistically significant (LME, p=0.04).

Specifically, having a CSF Aβ42 value below the cut-off was associated with a more rapid decline; 2.9 (SE 1.1) points difference per year, compared to those with a normal CSF Aβ42 value (p=0.0079) (Figure 6). The Aβ42 positive group was older (74.09 years) than those with normal Aβ42 values (69.48 years) (p=0.006), but this difference was adjusted for in the LME analysis. Patients with CSF T-tau values above the cut-off presented a more rapid cognitive decline compared to those with normal T-tau (2.0 (SE 1.1) points difference/year), but this result was not statistically significant (p=0.06). P-tau did not show any association with rate of decline. Table 9 summarize these results. We conducted these analyses in the subgroup of patients who started cholinesterase inhibitor treatment after baseline and found the same results (data not shown), which indicates that difference in medication status did not influence the data. To further explore the validity of the findings, we conducted an analysis only in the 24 patients with decreased DaTSCAN uptake. Again, the findings were similar to those in the full data set.

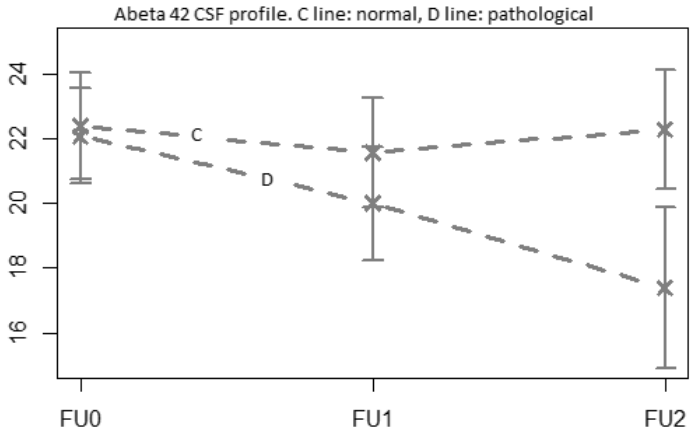


Figure 6. Change in MMSE score from baseline (FU0) to one (FU1) and two (FU2) years follow-up in those with (n=69) and without an abnormally low (n=31) CSF Aβ42 value
The difference was statistically significant (LME, p=0.0079).

Table 9. Annual rate of decline on MMSE in patients based on abnormal or normal values on the CSF markers

		Mean rate of change	*P* value
AD CSF profile	Pathological Normal	3.6 1.4	0.04
Aβ42 CSF	Pathological Normal	3.2 0.2	0.0079
T-tau CSF	Pathological Normal	3.5 1.5	0.06
P-tau CSF	Pathological Normal	2.2 2.5	0.85
The *P* value represents significance of the rate being different from the rate in the normal group			

5.3 The heterogeneity within DLB

These results have been sent for publication to Alzheimer´s Research & Therapy journal in September 2021.

5.3.1 Sample features

Here we included 107 probable DLB patients. The key characteristics of the cohort are shown in Table 10. The average age was 68±9 years and 28% of the patients was female. The average MMSE score was 25±4. Parkinsonism and fluctuating cognition were the most frequently reported clinical features (81% and 84%, respectively). Regarding the AD CSF biomarkers profile, 11% of the patients had AD-related pathology, 18% had an AD pathological change and 24% had amyloid-independent tau pathology. Atrophy was more frequent in parietal lobe (57%) than in medial temporal (33%) and frontal (39%) lobes. Table 10 shows key demographic and clinical data of the whole cohort, as well as CSF and MRI measures for all clusters.

Table 10. Characteristics of the whole cohort and DLB clusters

	Whole cohort (n=107)	Cluster 1 (n=39)	Cluster 2 (n=25)	Cluster 3 (n=24)	Cluster 4 (n=19)	Between-cluster ANOVA (*p*-value)
Age	68 (± 8.7)	70 (± 7.2)[b,d]	64 (± 7.7)[a,c]	71 (± 10)[a,d]	64 (± 6.9)[a,c]	0.001
Sex, n men (%)	77 (72.0%)	28 (71.8%)	21 (84.0%)[d]	21 (87.5%)[d]	7 (36.8%)[b,c]	0.001
Education, years mean (SD)	11 (± 3.8)	11 (± 2.8)[b,d]	8.2 (± 2.4)[a,c,d]	12 (± 3.5)[a,d]	15 (± 3.3)[a,b,c]	<0.001
Disease duration, years mean (SD)	4.3 (± 3.8)	4.2 (± 4.9)[d]	3.7 (± 2.7)[d]	3.5 (± 2.3)[d]	6.3 (± 3.8)[a,b,c]	0.013
MMSE score, mean (SD)	25 (± 4.0)	24 (± 3.9)[d]	22 (± 3.9)[d]	25 (± 3.8)[d]	28 (± 2.0)[a,b,c]	<0.001
Core clinical features						
Parkinsonism, n present (%)	87 (81 %)	33 (85 %)[c]	25 (100%)[c]	11 (46 %)[a,b,d]	18 (95 %)[c]	<0.001
Visual hallucinations, n present (%)	68 (64 %)	29 (74 %)[b]	8 (32 %)[a]	17 (71 %)	14 (74 %)	0.003
Fluctuating cognition, n present (%)	90 (84 %)	39 (100 %)[b]	12 (48 %)[a,d]	20 (83 %)	19 (100 %)[b]	<0.001
Probable RBD, n present (%)	68 (64 %)	23 (59 %)	20 (80 %)	15 (62 %)	10 (53 %)	0.234
CSF biomarkers						
Aβ-42, n abnormal (%)	31 (29 %)	16 (41 %)	6 (24 %)	8 (33 %)	1 (5 %)	0.037 *
Total tau, n abnormal (%)	23 (21 %)	4 (10 %)[c]	0 (0 %)[c]	19 (79 %)[a,b,d]	0 (0 %)[c]	<0.001
p-tau, n abnormal (%)	38 (36 %)	11 (28 %)[c]	4 (16 %)[c]	23 (96 %)[a,b,d]	0 (0 %)[c]	<0.001
AD CSF profile, n abnormal (%)						<0.001
AD pathology	12 (11 %)	3 (8 %)	1 (4 %)	8 (33 %)	0 (0 %)	
AD pathological change	19 (18 %)	13 (33 %)	5 (20%)	0 (0 %)	1 (5%)	
Amyloid independent tau-pathology	26 (24 %)	8 (21 %)	3 (12 %)	15 (63 %)	0 (0 %)	
Normal	50 (47 %)	15 (38 %)	16 (64 %)	1 (4 %)	18 (95 %)	
Visual rating scales						
MTA, n abnormal (%)	35 (33 %)	23 (59 %)[b,c]	5 (20 %)[a]	3 (12 %)[a]	4 (21 %)	<0.001
GCA-F, n abnormal (%)	42 (39 %)	20 (51 %)[d]	11 (44 %)[d]	11 (46 %)[d]	0 (0 %)[a,b,c]	0.002
PA, n abnormal (%)	61 (57 %)	19 (49 %)	19 (76 %)[d]	19 (79 %)[d]	4 (21 %)[b,c]	<0.001
Fazekas, n high WMH burden (%)	29/92 (32%) §	15/32 (47%)[d]	6/24 (25%)	7/18 (39%)	1/17 (6%)[a]	0.018

No missing data was recorded for the rest of the variables. a $p<0.05$ compared to cluster 1. b $p<0.05$ compared to cluster 2. c $p<0.05$ compared to cluster 3. d $p<0.05$ compared to cluster 4. § Available data for Fazekas is n = 92. * Does not survive the Hochberg's correction in post-hoc pair-wise comparisons. ANOVA: analysis of variance. MMSE: Mini-Mental state examination. Aβ: amyloid-β. p-tau: phosphorylated tau. AD: Alzheimer's disease. MTA: medial temporal lobe atrophy. GCA-F: frontal brain atrophy. PA: posterior brain atrophy. na: non applicable.

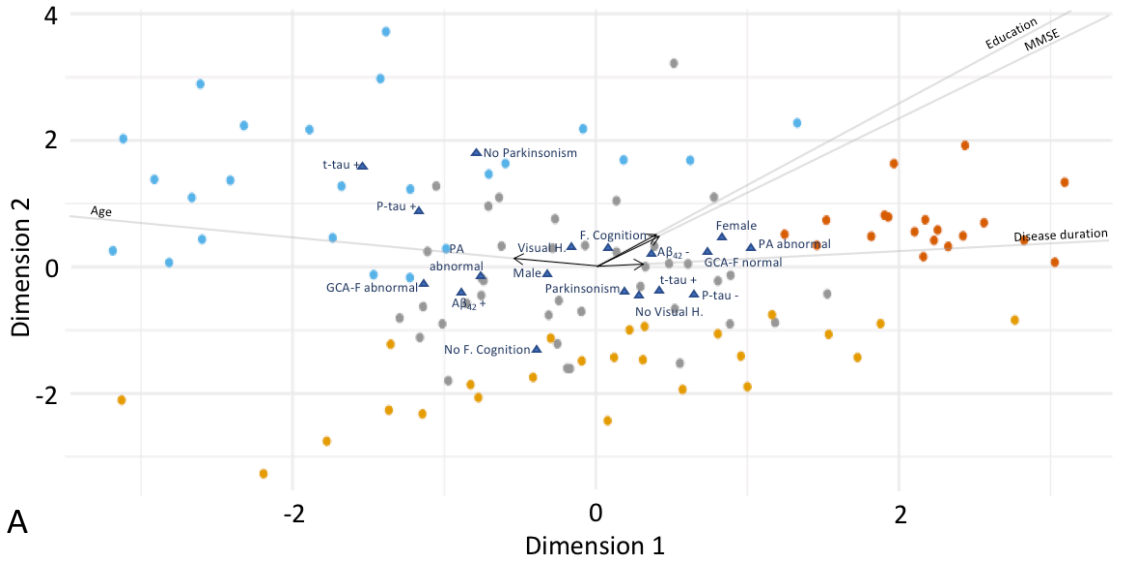
5.3.2 Factorial analysis of mixed data (FAMD)

The FAMD model identified three dimensions that together explained 38% of the variance in the data. Table 11 shows variables' contribution to these dimensions, and Figure 7 displays the three dimensions pair-wise. The first dimension accounted for 15.7% of the variance and was mostly driven by atrophy in frontal and parietal lobes, CSF p-tau levels, and age. In particular, older patients had increased atrophy in frontal and parietal lobes, and more often had abnormal CSF p-tau levels. In addition, CSF T-tau levels, MMSE, years of education, CSF Aβ42 levels, sex, disease duration, and parkinsonism also contributed statistically significantly to the first dimension.

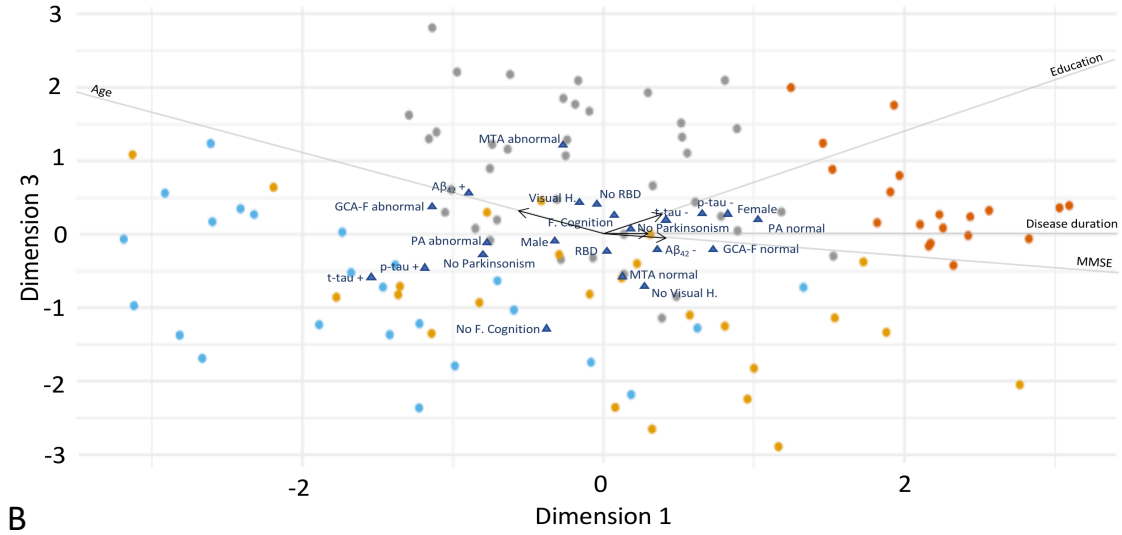
Table 11. Contribution of each variable to the dimensions of the FAMD

Variables	Dimension 1 (R^2 = 15.7%)	Dimension 2 (R^2 = 12.5%)	Dimension 3 (R^2 = 9.7%)
Age	13.02	0.87	6.42
Education	6.63	14.83	4.68
MMSE	7.32	12.70	0.31
Disease duration	4.27	0.08	0.00
Sex	4.67	2.29	1.27
CSF Aβ-42	5.76	2.14	5.74
CSF total tau	11.49	18.05	4.71
CSF p-tau	13.66	11.10	5.49
Parkinsonism	2.59	19.46	0.89
Visual hallucinations	0.78	3.76	14.02
Cognitive fluctuations	0.49	9.17	15.13
Probable RBD	0.02	1.52	4.31
MTA	0.61	1.04	31.93
GCA-F	14.72	1.76	4.09
PA	13.97	1.22	1.00

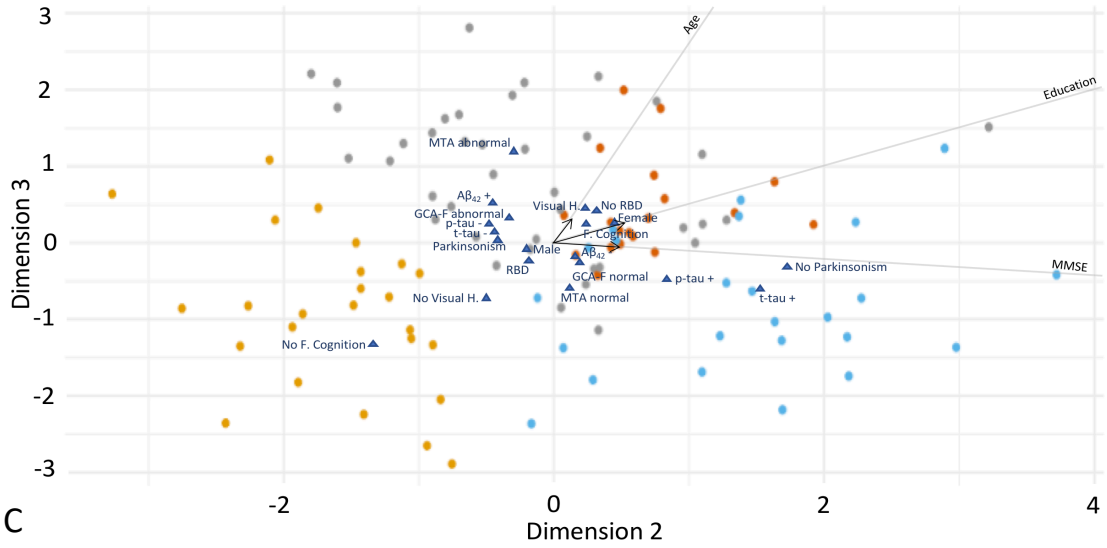
Values represent the percentage of contribution of each variable to the total variation captured by each dimension. MMSE: Mini-Mental State examination; CSF: cerebrospinal fluid; Aβ: amyloid-beta; p-tau: phosphorylated tau; MTA: medial temporal lobe atrophy; GCA-F: frontal brain atrophy; PA: posterior brain atrophy; FAMD: factorial analysis of mixed data.



A) Dimension 1 vs. dimension 2



B) Dimension 1 vs. dimension 3



C) Dimension 2 vs. dimension 3

Figure 7. Dimensions from the FAMD model (separate plots for continuous and categorical variables)

The figure displays the three dimensions from the FAMD model, pair-wise.
Continuous variables are depicted as arrows projecting lines (arrows represent the direction and degree of contributions). Categorical variables are depicted as triangles, which reflect variables' centroids in the different levels of categorical variables. MMSE: Mini-Mental State examination; F Cognition: fluctuating cognition; Visual H: visual hallucinations; RBD: REM sleep behavior disorder; Aβ42: amyloid-β42; p-tau: phosphorylated tau; T-tau: total tau; MTA: medial temporal lobe atrophy; GCA-F: frontal brain atrophy; PA: posterior brain atrophy.

The second dimension accounted for 12.5% of the variance and was mostly driven by parkinsonism, CSF total tau levels, years of education, and MMSE. Patients with higher education showed higher MMSE scores despite more frequently having abnormal CSF total tau levels, and they had a lower frequency of parkinsonism. In addition, CSF p-tau levels cognitive fluctuations, visual hallucinations, sex, and CSF Aβ42 levels also contributed statistically significantly to the second dimension.

The third dimension explained 9.7% of the variance and was mostly driven by atrophy in medial temporal lobes, cognitive fluctuations, and visual hallucinations. Patients with atrophy in medial temporal lobes more often had cognitive fluctuations and visual hallucinations. In addition, age, CSF Aβ42, p-tau, and total tau levels, as well as years of education, probable RBD, and atrophy in frontal lobes also contributed statistically significantly to the third dimension.

5.3.3 Hierarchical clustering analysis

Subsequently, we clustered the patients using agglomerative hierarchical clustering analysis based on the three dimensions from the FAMD model as input variables. Calinski-Harabasz values showed that four clusters (CH = 44.5) were more appropriate than two, three, or five clusters (CH < 42.0). Figure 8A shows the dendrogram from the cluster analysis, and Figure 8B displays the distribution of the DLB patients colored by clusters 1 to 4.

Cluster one (C1) included 37% of the patients (n = 39), cluster two (C2) included 23% (n = 25), cluster three (C3) included 22% (n = 24), and cluster four (C4) included 18% (n = 19) of the DLB patients.

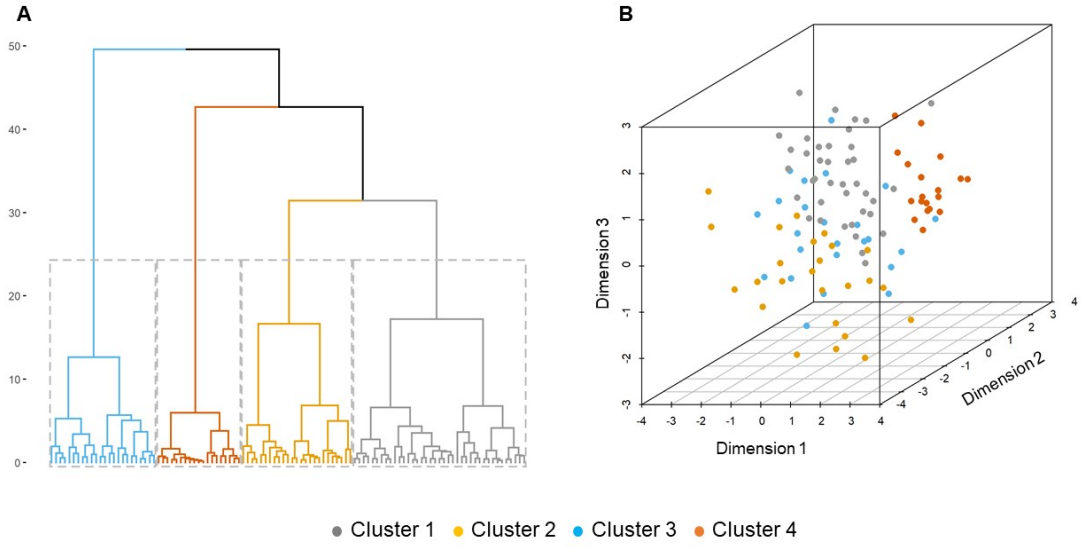


Figure 8. Dendrogram and clusters from the agglomerative hierarchical cluster analysis

A) Dendrogram from the cluster analysis, with DLB patients depicted on the x-axis (each lower branch is a patient) and similarity depicted on the y axis (the shorter the distance along the axis, the greater the similarity). B) Three-dimensional space generated by dimensions 1, 2, and 3 from the FAMD model. Dots represents the DLB patients colored by cluster (1 to 4) and distributed across the three-dimensional space.

Patients in C1 were among the oldest and had intermediate levels of education, disease duration, and MMSE scores. Further, all the patients in C1 had cognitive fluctuations. Regarding AD CSF biomarkers, C1 had the highest frequency of an AD pathological change (abnormal levels of CSF Aβ42 alone). As for regional brain atrophy and WMH, patients in

C1 had the highest frequency of medial temporal atrophy and high WMH burden. In addition, these patients showed intermediate levels of parietal atrophy.

Patients in C2 had the lowest levels of education, MMSE scores, and frequency of visual hallucinations and cognitive fluctuations; and were among the clusters with younger age and shortest disease duration. Patients in C2 had the highest frequency of parietal atrophy, together with patients in C3.

Patients in C3 were the oldest, had intermediate levels of education and MMSE scores, had the shortest disease duration, and were the patients with lowest frequency of parkinsonism. Further, patients in C3 had the highest frequency of abnormal CSF levels of p-tau, either in combination with a abnormal Aβ42 biomarker (AD pathology), or independently of Aβ42 (amyloid-independent tau-pathology). Additionally, C3 patients had a significantly higher frequency of abnormal levels of T-tau in CSF.

Patients in C4 were among the youngest, had the lowest frequency of men, had the highest levels of education and MMSE scores, and had the longest disease duration. All patients in C4 had cognitive fluctuations. All patients but one had a normal CSF AD biomarker profile. Furthermore, patients in C4 had the lowest frequency of parietal atrophy and WMH burden, and none of them had frontal atrophy.

Clusters did not significantly differ in the frequency of probable RBD or abnormal CSF levels of Aβ42. Yet, the difference in abnormal levels of amyloid-β emerged with its combination in AD CSF profiles (AD pathology, AD pathological change and amyloid-independent tau-pathology), likely due to the contribution of tau-related pathology.

5.4 Global summary of the results

In this doctoral thesis we have investigated the interaction of two neurodegenerative proteinopathies: DLB and AD. For this purpose, we analyzed demographic, clinical features, global cognition, regional brain atrophy patterns and CSF levels of AD biomarkers in patients with DLB from the E-DLB cohort.

We explored the impact of AD-related pathology on regional brain atrophy and longitudinal cognitive decline in patients with DLB. The combined results from these analysis showed that pathological CSF levels of Aβ42 were associated with atrophy in the medial temporal and posterior cortices. Further, reduced levels of CSF Aβ42 were related to a more rapid cognitive decline. On another hand, pathological CSF levels of p-tau were associated with PA, yet they did not correlate with cognitive worsening. Further, CSF levels of T-tau were neither related to patterns of regional brain atrophy nor cognitive decline; and none of the AD CSF biomarkers contributed to the prediction of frontal lobe atrophy.

In addition to the individual contribution of AD CSF biomarkers, we assessed their combined influence in cognitive decline. Hence, we defined an AD CSF profile as the presence of pathological (i. e. low) CSF values of Aβ42 plus pathological (.i e. high) values of p-tau or T-tau, because this analysis was conducted before the publication of the AT(N) classification system for AD biomarkers in 2018 (122). Thus, we found that DLB patients with an AD CSF profile presented a faster cognitive decline. Moreover, male gender and higher level of education were both associated with a more rapid decline.

Finally, we parsed DLB heterogeneity of biological, clinical and demographic data using factorial analysis (FAMD) and multimodal clustering. In the FAMD, we identified three dimensions that explained 38% of the variance. CSF levels of p-tau and T-tau were among the most important drivers of dimensions 1 and 2, respectively. Moreover, CSF levels of Aβ42 contributed statistically significantly to the three dimensions. Based on these three dimensions, we clustered the patients using agglomerative hierarchical clustering analysis. Consequently, we obtained four clusters that ranged from on subgroup with almost normal AD CSF biomarkers (cluster 4) to three subgroups with various degrees of AD-related pathology: cluster 1 had the highest frequency of AD pathological change (i. e. only Aβ42 positive), cluster 3 presented the highest frequency of tau pathology, either in combination with abnormal CSF levels of Aβ42 or in isolation (amyloid-independent tau pathology); whereas cluster 2 was an "intermediate" subgroup between the former two clusters. Furthermore, the clusters showed distinct demographic, clinical and regional brain atrophy profiles. Still, they did not differ significantly in the frequency of probable RBD or abnormal levels of Aβ42. In summary, DLB patients are heterogeneous and four endophenotypes were identified with distinctive biological, clinical and demographic profiles.

6. Discussion

A significant proportion of DLB patient exhibit concomitant AD pathology (4,100,108,138–140) and the degree of AD copathology is moderate and severe in up to 70% of these patients (64,101,102,141) In this thesis, we have investigated how these proteinopathies interact resulting in regional brain atrophy, predicting cognitive decline, and generating different phenotypes.

Clinical diagnosis of DLB and AD dementia require the fulfilment of consensus criteria with two different levels of certainty ranging from probable: when there is no other neurodegenerative disease that explains the cognitive deficits, to possible if there is(are) other(s) suspected concomitant pathology(ies) (29). But this pragmatic approach, conflicts with a reality shown by neuropathological studies indicating that a high percentage of patients with a parkinsonian syndrome might present multiple comorbidities (139). This reality is far more complex than the presence of one pathology justifying the clinical picture, and it seems that the "pure" phenotypes are the exception, not the norm. Therefore, it is of high importance to study mixed neurodegenerative diseases to better understand the interaction of different pathologies and their influence in the clinical and biological features.

AD biomarkers helps us to investigate in vivo how AD-related pathology influences DLB patients. Thus, we have demonstrated that DLB patients with coexisting AD-related pathology have a distinctive brain atrophy pattern, present a faster cognitive decline, and can be divided into different endophenotypes.

These results have several implications. First, the indication that DLB patients with AD pathological change (amyloidosis) have worse cognitive performance over time suggests a poorer prognosis in the clinical setting, and this information might be important to patients and their families to better understand the evolution of the disease, and plan their future. Second, the influence of AD-related pathology on brain atrophy has unraveled a possible specific tau distribution in DLB that is different from the proposed Braak and Braak stages. Third, the increased heterogeneity among patients with DLB and concurrent AD-related pathology demonstrates a higher complexity that poses more difficulties for accurate diagnosis in the clinical practice, and can explain why DLB is still an underrecognized disorder. Finally, we speculate that since there is a high proportion of DLB patients with

overlapping AD-related pathology, disease modifying treatments (DMTs) targeting amyloid-β or tau could be of interest in the therapeutic approaches that could be tested in patients with DLB.

In the following sections we discuss in detail the influence of AD-related pathology in DLB patients.

6.1 Contribution of AD-related pathology to regional brain atrophy in DLB

Patterns of regional brain atrophy helps us to differentiate DLB from AD, since DLB patients exhibit less overall atrophy than AD, due to less MTA but similar rates of PA, and GCA-F (142) In fact, preserved medial temporal lobe structure on MRI is a supportive biomarker for the diagnosis of DLB (29).

But what are the underlying mechanisms of neurodegeneration that result in these patterns of brain atrophy? One way to analyse this question is to investigate how AD-related pathology contributes to regional brain atrophy in DLB patients. Our findings suggest that amyloid and tau-related pathologies have a synergistic effect in posterior cortex atrophy, whereas amyloid-related pathology is associated with atrophy in the medial temporal lobe.

Previous studies using PET and MRI neuroimaging in DLB patients have shown similar results. Thus, higher amyloid-β burden has been associated the atrophy in the medial temporal lobe structures (143–145) With regard to the posterior cortex, in the study conducted by Sarro et al, incresead amyloid-β burden measured with ^{11}C-Pittsburgh compound B was associated with greater atrophy in posterior cingulate gyrus, and occipital lobe (143) Likewise, greater tau load measured with ^{18}F-AV-1451 has been found in parietal lobes (146) as well as posterior and inferior temporoparietal, and occipital lobes (147).

Our results indicate that DLB patients present a diffuse distribution of amyloid plaques that resembles that of AD, but have a distinctive localization of neurofibrillary tangle (NFT) pathology. To explain these findings, we especulate that amyloid-β and tau pathologies might have a combined effect in posterior cortex neurodegeneration. Similar results have been demonstrated by Kantarci and colleagues (147). These authors performed MRI, amyloid and tau PET neuroimaging in DLB patients to determine the pattern of tau

deposition and its relationship with amyloid-β burden when compared with AD patients. Their results showed that increased tau burden in posterior brain areas (posterior temporoparietal and occipital cortices) correlated with higher global amyloid load in DLB. These findings are consistent with an autopsy confirmed study where the highest burden of tau related pathology was found in the occipital lobes of DLB patients (148) But, why in DLB tau pathology has a different localization than expected according to Braak NFT distribution? Well, it is possible that this unique distribution of tau is influenced by Lewy body pathology. A recent neuropathological study that used digital histology to analyse the impact of co-ocurrence of AD in DLB patients found that anatomical distribution of tau pathology was similar to that of α-synuclein (149) probably due to the interaction of tau and α-synuclein by coseading, and promotion of each other accumulation (110,113).

Our original results help us to unravel in vivo underpinnings of neurodegeneration in DLB patients with overlapping AD pathology, which was possible thanks to the use of CSF biomarkers. Another study that investigated the relationship of AD CSF biomarkers and atrophy rates in DLB patients, also found that abnormal CSF levels of amyloid-β were associated with MTA (150). Additionally, the authors demonstrated that abnormal CSF levels of T-tau were associated with GCA-F and PA. Yet, our findings did not support a relationship between levels of CSF T-tau and brain atrophy in DLB patients. We explain our results based on the fact that CSF T-tau is a marker of global unspecific neurodegeneration, while visual rating scales indicate regional brain atrophy, but we cannot exclude the possibility that our findings are due to the low number of subjects with abnormal CSF levels of T-tau in our sample, as it has been found by other authors (151).

In relation to the frontal lobe, neither amyloid-β nor tau-related pathologies were associated with atrophy in this brain region in DLB. Similar results have been found in AD (127). In DLB, previous studies have demonstrated increased amyloid-β load in frontal regions (152,153) but this has not been associated with greater grey matter atrophy (143).

Another novelty of our study was use of random forest (classification) (131) as the statistical method to: 1) investigate the combined effect of AD CSF biomarkers on regional brain atrophy measured with visual rating scales, 2) model the effect of age, sex, level of education and disease duration on the association between AD CSF biomarkers, and regional brain

atrophy; and 3) analyse the predictive power of all these variables in combination as predictors of regional brain atrophy.

The usage of this machine learning technique revelead interesting results. As aforementioned, we found an association between amyloid-β and MTA, but the classification error for the prediction of patients with abnormal MTA scores was higher than the classification error for the prediction of normal MTA scores. This indicates that in the abscense of amyloid-β is unlikely to find MTA, yet when MTA is present is not always related to amyloid pathology. Therefore, there could be other factors contributing to MTA such as other copathologies (i. e. TDP-43 or hippocampal sclerosis). Similarly, the classification error for the prediction of patients with normal and abnormal PA scores were high in our model, although under the threshold of error by chance. Hence, we acknowledge that the association between PA and both amyloid-β and tau-related pathologies is a preliminary finding that should be replicated in future studies.

Additionally, random forest classification models permitted us to study to what extent age, sex, education, and disease duration contribute to regional brain atrophy in combination with AD CSF biomarkers, because it is unknown wheter these variables should be investigated as confounding or contributing factors to regional brain atrophy in neurodegenerative diseases (154) Thus, we found that atrophy in the medial temporal and posterior cortices was associated with shorter disease duration, and older age. Graff-Radford et al have shown similar results where DLB patients with lower hippocampal volume have shorter survival (155) On another hand, we found that atrophy in the frontal lobe was associated with older age, male sex, and lower level of education. These findings have potential predictive value in clinical practice and could help to identify patients with worse prognosis. We were able to demonstrate these associations by using a multivariate analysis, which has helped us exposed the effect of variables such as disease duration, that cannot be captured in univariate or bivariate models, or that are removed in models testing for partial effects (132) A further interesting result, was that the Pearson correlation between levels of CSF p-tau and PA scores was not significant, yet the levels of CSF p-tau contributed to the prediction of PA in the multivariate random forest model. This indicates that the effect of tau-related pathology on posterior brain atrophy is not direct, but emerges in the presence of amyloid-β related pathology, suggesting a synergistic effect of AD-related pathology in the prediction of atrophy in the posterior cortex. Other contributing factors in the random forest for PA were

age, level of education, and disease duration. We propose that overlapping AD pathology in DLB have a stronger impact in the integrity of the posterior brain cortex in older subjects with less education, which could result in a shorter disease duration.

6.2 Influence of AD-related pathology in DLB cognitive decline

Results from neuropathological studies indicate that DLB patients with mixed AD pathology have worse cognitive performance (156), greater memory impairment, poorer visuospatial performance and faster cognitive decline, whereas pure DLB patients present worse performance in executive function and attention (157,158).

Similar findings have been demonstrated in PD patients where lower levels of CSF Aβ42 have been associated with more rapid cognitive decline (159–161) Likewise, higher CSF levels of p-tau have been found to predict worse performance in memory and executive functions in PD (162) and are associated with impaired recognition and naming in PDD (163).

In DLB patients, an AD CSF profile have been associated with worse performance in memory and orientation (164) being amyloid an independent predictor of cognitive performance (165,166).

Yet, little is known about the impact of AD concurrent pathology on longitudinal cognitive performance of DLB patients. We have demonstrated that CSF Aβ42 levels predict a faster cognitive decline in DLB.

Similar results have been suggested by neuroimaging studies with FDG and amyloid PET, indicating that concomitant AD pathology in DLB is associated with worse cognitive impairment over time (167,168).

However, studies that have analyzed AD CSF biomarkers and cognitive progression in DLB patients are scarce. The few studies that have investigated this issue, have demonstrated an association of pathological levels of amyloid-β in CSF with lower MMSE at baseline and worse performance in the memory domain, but not with a more rapid cognitive decline (105,169). Therefore, our findings need further investigation.

We have not found a correlation with CSF T-tau o p-tau levels with progressive cognitive impairment. This is interesting because in AD, tau-related pathology is associated with cognitive worsening, but in DLB it seems that amyloid-β plays a more important role in the evolution of the cognitive deficits.

We did not investigated α-synuclein impact on the longitudinal cognitive impairment, because there is no reliable biomarker available. However, results from animal models suggest a synergistic interaction between amyloid-β, tau, and α-synuclein that favors their aggregation resulting in a faster cognitive decline (170).

Our results are relevant because they have prognostic value, and could be used in the clinical practice to inform patients and their families about the progression of the disease. Additionally, these patients may have a distinctive response to disease modyfing treatments (DMTs); and since amyloid is altered in a percentage of DLB patients, they could be elegible for anti-amyloid treatments, currently approved or under investigation in AD. Moreover, if an anti-amyloid therapy is tested in clinical trials with DLB patients who have AD concomitant pathology, our results support the use of amyloid-β as a potential biomarker for disease progression and/or treatment monitoring.

6.3 The heterogeneity within DLB and the impact of AD-related pathology

DLB diagnosis can be challenging because not all patients exhibit all core features, and they can manifest at different time points in the course of the disease. This increases clinical heterogeneity and partly explains why this disease is underdiagnosed.

Previous studies have investigated DLB heterogeneity, finding distinct clinical subtypes (171) that can be identified from the initial presentation (172) Nevertheless, investigating the biological heterogeneity within DLB will increase our current understanding of these endophenotypes. To this end, we parse DLB heterogeneity by using multimodal subtyping method to identify subpopulations of patients with common demographic, clinical, MRI, and AD CSF profiles. We found that DLB patients are an heterogeneous group resulting from the combination of demographic and clinical features, as well as regional brain atrophy patterns and concomitant AD-related pathology.

Our findings suggest that the biological heterogeneity in DLB could be partly explained by the coexistence of AD-related pathology. Hence, we identified four DLB endophenotypes ranging from virtually none (cluster 4) to various degrees of concomitant AD-related pathology (clusters 1 to 3). Interestingly, these subgroups exhibited distinct regional brain atrophy, clinical and demographic features.

The "purest" DLB endophenotype was cluster 4 because it had almost normal AD CSF biomarkers, and very low burden of cerebrovascular disease. Therefore, we suspect that the underlying pathology in this subgroup is α-synuclein-related. This cluster was also the most "benign" given its younger age, longer disease duration, higher MMSE scores, least regional brain atrophy, and normal CSF levels of T-tau. Similar findings have been reported previously, where "pure" DLB patients were younger, and had better global cognition (105).

The other three endophenotypes showed various degrees of concomitant AD-related pathology, resulting in distintive demographic, clinical and regional brain atrophy profiles. AD pathological change was most frequently found in clusters 1 and 2. Yet, cluster 1 was characterized by MTA, and cluster 2 by PA. On another hand, the highest frequency of tau-related pathology and PA was demonstrated in cluster 3. These results support our previous findings of the association between AD CSF biomarkers and regional brain atrophy, where amyloid-β related pathology was associated with MTA, while tau-related pathology was related to PA.

In addition to MTA, cluster 1 was also characterized by older age and high burden of cerebrovascular disease. Similarly, previous studies have demostrated an association between amyloid-β and older age (165), atrophy in the medial temporal lobe (117,143,145,150), and cerebral amyloid angiopathy (173,174). Here we have been able to identify a subgroup of DLB patients that combines all these features.

Another hypothesis generated by our data is the possible association between tau-related pathology and disease duration in DLB. Cluster 3, the subgroup with highest frequency of tau-related pathology, was among the clusters with shortest disease duration. Previous studies have shown that tau pathology is associated with worse prognosis in DLB (64). Similarly, cluster 2 was the other phenotype with a short disease duration. Nevertheless, this cluster showed a low frequency of tau-related pathology. Other features of this subgroup were younger age, low level of education (i. e. lower cognitive reserve), and low MMSE

scores. Hence, we propose that lower levels of tau pathology may be enough to lead to low MMSE scores in shorter time, at younger ages, as we have found in cluster 2.

Altogether, disease duration was the shortest in both cluster 2 and 3, the two clusters with greater posterior brain atrophy. This might suggest a more aggressive presentation of the disease. Likewise, the AD subtype with greater atrophy in the posterior cortex has been proposed as the most aggressive presentation of the disease, possibly due to a higher frequency of mixed AD and Lewy body pathology (154).

Furthermore, the presence of concomitant AD-related pathology influenced the clinical features of the DLB subgroups. Previous studies have shown that concomitant AD pathology in DLB result in a less typical presentation (175,176) possibly because these patients have lower frequency of core features (177) Similary, our research group have demonstrated that patients with DLB and tau-related pathology have lower number of concurrent core features (178). These findings are clinically relevant because they suggest that patients with mixed DLB and AD have a higher risk of misdiagnose.

Interestingly, we found that the frequency of RBD was not different across clusters. Few studies have investigated the impact of AD or cerebrovascular pathologies upon probable RBD. Autopsy confirmed studies have suggested that patients with a clinical history of RBD have less AD-related pathology and a higher frequency of diffuse Lewy body disease (179–181). Additionally, recent biomarker studies have found that higher tau and cerebrovascular burden -but not of amyloid-β- were associated with a lower frequency of probable RBD (165,182). Still, it seems that RBD prevalence is similar among distinct DLB phenotypes, but this finding needs to be replicated in future studies.

Another interesting result was that CSF levels of amyloid-β were not one of the main drivers of the dimensions in the FAMD model. Contrary of having no influence in the heterogeneity, it suggests that amyloid-β could be an underlying factor in all dimensions, contributing to more than one dimension at the same time. Further, it is widely known that amyloid-β has a weaker contribution to brain atrophy and cognitive impairment compared to tau pathology (183–185).

6.4 Limitations

The results presented in this doctoral thesis have some limitations. First, we have analized retrospective data from different centers across Europe. However, using data from multiple centers with extensive expertise in standardized criteria, and methods to diagnose neurodegenerative diseases is a strength because the findings are easier to generalize. Second, instead of using continuous values for CSF or quantitative measures for MRI data, we have used cut-off values to dichotomize CSF levels of AD biomarkers, and rating scales to investigate regional brain atrophy. These approaches were used to minimize methodological differences across sites. Third, we have used clinical criteria for the diagnosis of probable DLB without autopsy confirmation, and only a subset of patients had available DaTSCAN. Nonetheless, the clinical criteria have high specificity, which suggests that 80% to 90% of patients who fulfill clinical criteria for probable DLB do also pathological criteria (186).

Finally, there are specific limitations from in each analysis. In the analysis of regional brain atrophy and heterogeneity we used a cross-sectional design, and the interval between MRI and CSF collection was long in 15.11% of cases (ranging from 3 to 12 months). Also, although random forest analysis can manage multicollinearity in some degree, it can result in an underestimation of the contribution of multicollinear variables. On another hand, in the analysis of longitudinal cognitive decline, the follow up period was relatively short, so it is recommend to perform studies with longer duration, partly because fluctuating cognition in DLB patients can mask possible associations between cognitive performance and CSF biomarkers. And in the study of heterogeneity, we used a data-driven approach, hence the results should be considered hypothesis generating; and we cannot rule out the possibility that part of the heterogeneity investigated is due to differences among centers.

7. Conclusions

1. Abnormal levels of CSF Aβ42 and p-tau contribute in combination to posterior brain atrophy, and reduced CSF levels of Aβ42 are associated with atrophy in the medial temporal lobe in patients with DLB.

2. DLB patients with abnormal levels of CSF Aβ42, present a faster cognitive decline during 2 years of follow up. Additionally, CSF levels of total and p-tau in DLB patients are not associated with longitudinal cognitive decline over time.

3. DLB is an heterogeneous disease conformed by four endophenotypes with distinct demographic, clinical, regional brain atrophy patterns, and AD CSF biomarkers profiles.

8. Future lines

In this thesis, we have layout the influence of AD concomitant pathology in the regional brain atrophy, longitudinal cognitive decline, and heterogeneity of patients with DLB, thanks to the availability of biomarkers for AD. Nevertheless, there is no reliable biomarker available for α-synuclein. New approaches for the identification of α-synuclein in biofluids -such as RT-QuIC- have shown encouraging results, but more studies are needed to validate these promising findings. It would be interesting to investigate the impact of AD copathology in DLB by combining amyloid-β, tau, and α-synuclein biomarkers.

Likewise, is necessary to consider the potential role of other proteinopathies such as TDP-43 (for which biomarker is also lacking), as well as biological processes like neuroinflammation, synaptic pathology, or axonal injury. New techniques like proximity extension assay (PEA) technology for the identification of fluid biomarkers (187,188) or novel imaging PET tracers (189), could help to unravel the interplay of different proteinopathies and biological processes in the diagnosis, clinical presentation, and disease progression of DLB. Ideally, such future studies should take advantage of multimodal approaches and be conducted in large longitudinal cohorts, given the complexity of the problem at hand. This is why is important to promote collaboration across different research groups, as it is been done by the E-DLB.

Potential studies beyond the scope of this thesis are the analysis of disease progression, and the characterization of the neuropsychological profiles of the DLB endophenotypes we found; as well as the longitudinal study of regional brain atrophy in DLB with concomitant AD pathology.

Another interesting question is to understand at what level of abnormality a copathology starts being *pathological*, that is to determine the cut-off of AD biomarkers in DLB. Until now, CSF and PET studies of AD biomarkers in DLB, often use the cut-offs established for AD to analyse the influence of amyloid-β and tau pathologies. But it might be that DLB has a different pathological threshold in which these proteins affect the clinical presentation, cognitive performance and brain atrophy.

Finally, the case has been made about the possible eligibility of DLB patients with mixed AD pathology for AD DMTs such as anti-amyloid therapies. To this end, clinical trials in

this population should be conducted, and AD biomarkers can be used to stratify DLB patients and increase statistical power (190). In addition to investigating the potential benefit of AD DMTs in DLB; other questions like the possible influence of AD-related pathology on the response to future α-synuclein therapies could be explored.

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10. Annexes

Annex I: Supplementary tables

Supplementary table 1: Overview of CSF procedures per center

Center	Centrifuging	Storage	Analysis Essay	Cut off values [ng/L]
Mälmo	Centrifuged at 2000g for 10 minutes at 4°C	Stored at -80°C	INNOTEST Double sandwich ELISAs	Aβ42: 500 t-tau: <50 years old: 300 50-70 years old: 450 >70 years old: 500 p-tau: 60
Strasbourg	Centrifuged at 1000g for 10 minutes at 4°C	Stored at -80°C	INNOTEST Double sandwich ELISAs	Aβ42: <550 T-tau: >400 P-tau: >80
Amterdam	Centrifuged at 1800 g for 10 min at 4°C	Aliquots of 0.5 mL stored in polypropylene tubes at -80°C	INNOTEST Double sandwich ELISAs	$A\beta_{42}$: <550 t-tau: >375 p-tau: >52
Stockholm	Centrifuged at 2000g for 10 minutes at 4°C	Aliquots of 0.5mL of 1mL stored in polypropylene tubes at -80°C	INNOTEST Double sandwich ELISAs	Aβ42: <550 T-tau: >400 P-tau: >80
Brescia	Centrifuged at 3000g for 3 minutes at 4°C	Stored in polypropylene tubes at -80°C	INNOTEST Aβ42, Tau and P181-tau	Aβ42: <650 T-tau: >400 P-tau: >30
Stavanger	Centrifuged at 2000g for 10 minutes at 4°C	Stored in polypropylene tubes at -80°C	Aβ42: Biosource Europe S.A. t-tau: INNOTEST hTau p-tau: INNOTEST Phos-pho-Tau (181)	Aβ42: <482 T-tau: >320 P-tau: >52
Ljubljana	Centrifuged at 4000g for 10 minutes at 2-4°C	Stored at -70°C	INNOTEST Double sandwich ELISAs	Aβ42: <550 T-tau: >400 P-tau: >80
Barcelona	Centrifuged at 2000g for 10 minutes at 4ºC	Stored in polypropylene tubes at -80ºC	INNOTEST Double sandwich ELISAs	Aβ42: <670 T-tau: >398 P-tau: >65

Supplementary table 2: Overview of MRI parameters per center

Center	MRI parameters	
Strasbourg	Scanner, Field strength(T), TR(ms), TE(ms), TI(ms), FA, NoA, Resolution(mm)	Siemens Verio, 3, 1900, 2.53, 900, 9, 1, (1, 1, 1)
Amsterdam	Scanner, Field strength (T), TR(ms), TE(ms), TI(ms), FA, NoA, Resolution(mm)	GE Signa, 3T, 8, 3, 450, 12, (0.98x0.98x1)
Stockholm	Scanner, Field Strength (T), Resolution(mm)	Siemens Aera, 1.5T, (1, 1, 1) mm resolution Siemens Avanto, 1.5T, (1.5, 1, 1) mm resolution Siemens Avanto, 1.5T, (1.2, 1, 1) mm resolution Siemens Avanto, 1.5T, (1.4, 1, 1) mm resolution Siemens Symphony, 1.5T, (1.5, 1, 1) mm resolution Siemens Symphony, 1.5T, (0.81, 1, 1) mm resolution Siemens Trio, 3T, (1, 1, 1) mm resolution Siemens Trio, 3T, (1.2, 1, 1) mm resolution Siemens Trio, 3T, (0.9, 1, 1) mm resolution Siemens Trio, 3T, (1.4, 1, 1) mm resolution
Brescia	Scanner, Field strength(T), TR(ms), TE(ms), TI(ms), FA, NoA, Resolution(mm)	Siemens Avanto, 1.5, 2050, 2.56, 1100, 15, 1, (0.5, 0.5, 1)
Stavanger	Scanner, Field strength(T), TR(ms), TE(ms), FA, NoA, Resolution(mm)	Philips Intera, 1.5, 10, 4.6, 30, 2, (1.01, 1.01, 1) Philips Intera, 1.5, 20, 16, 30, 1, (1.02, 1.02, 1) GE Signa Excite, 1.5, 8.224, 3.144, 7, 1, (1, 1, 1)
Ljubljana	Scanner, TR(ms), TE(ms), FA, NoA, Resolution(mm)	Philips Achieva, 25, 4.60, 30, 1, (0.9375, 0.9375, 1) Siemens TrioTim, 2300, 4.71, 12, 1, (1, 1, 1) Philips Achieva, 7.27, 3,331, 8, 1, (1, 1, 1)
Barcelona	Scanner, Field strength (T), TR (ms), TE (ms), TI (ms), Resolution (mm)	Siemens Magnetom Aera, 3T, 2200ms, 2,23ms, 968ms, 1.1x1.1x1.2mm

Annex II: Article "The combined effect of amyloid-β and tau biomarkers on brain atrophy in dementia with Lewy bodies"

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The combined effect of amyloid-β and tau biomarkers on brain atrophy in dementia with Lewy bodies

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ABSTRACT

Background: Alzheimer's disease (AD)-related pathology is frequently found in patients with dementia with Lewy bodies (DLB). However, it is unknown how amyloid-β and tau-related pathologies influence neurodegeneration in DLB. Understanding the mechanisms underlying brain atrophy in DLB can improve our knowledge about disease progression, differential diagnosis, drug development and testing of anti-amyloid and anti-tau therapies in DLB.

Objectives: We aimed at investigating the combined effect of CSF amyloid-β42, phosphorylated tau and total tau on regional brain atrophy in DLB in the European DLB (E-DLB) cohort.

Methods: 86 probable DLB patients from the E-DLB cohort with CSF and MRI data were included. Random forest was used to analyze the association of CSF biomarkers (predictors) with visual rating scales for medial temporal lobe atrophy (MTA), posterior atrophy (PA) and global cortical atrophy scale-frontal subscale (GCA-F) (outcomes), including age, sex, education and disease duration as extra predictors.

Results: DLB patients with abnormal MTA scores had abnormal CSF Aβ42, shorter disease duration and older age. DLB patients with abnormal PA scores had abnormal levels of CSF Aβ42 and p-tau, older age, lower education and shorter disease duration. Abnormal GCA-F scores were associated with lower education, male sex, and older age, but not with any AD-related CSF biomarker.

Conclusions: This study shows preliminary data on the potential combined effect of amyloid-β and tau-related

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pathologies on the integrity of posterior brain cortices in DLB patients, whereas only amyloid-β seems to be related to MTA. Future availability of α-synuclein biomarkers will help us to understand the effect of α-synuclein and AD-related pathologies on brain integrity in DLB.

1. Introduction

Neuropathological studies have shown that many patients diagnosed with dementia with Lewy bodies (DLB) often have Alzheimer's disease (AD)-related pathology (Gomez-Isla et al., 1999; Schneider et al., 2009; Halliday et al., 2011; Dugger et al., 2014; Sierra et al., 2016) In these mixed cases, it has been found that the degree of AD-related pathology is moderate or severe in more than 70% of patients (Kosaka, 1990; Marui et al., 2004; Barker et al., 2002; Irwin et al., 2017). The combination of these proteinopathies have implications in the clinical phenotype. Thus, postmortem studies have shown that the coexistence of amyloid-β and tau-related pathologies in addition to the defining alpha-synuclein pathology usually results in a less typical presentation of DLB core features: a lower frequency of recurrent visual hallucinations, parkinsonism, REM sleep behavior disorder (RBD) and fluctuating cognition (Merdes et al., 2003; Del Ser et al., 2001; Tiraboschi et al., 2015; Compta et al., 2013; Murray et al., 2013), and a more severe disease course (Irwin et al., 2017; Howlett et al., 2015; Kraybill et al., 2005; Williams et al., 2006).

Similar findings have been obtained in vivo (Di Censo et al., 2020), which is more relevant for the earlier disease stages compared to the end-stage diseases assessed at autopsy. In this regard, cerebrospinal fluid (CSF) and neuroimaging studies have shown concomitant AD-related biomarkers in a significant proportion of DLB patients who often are older, have shorter disease duration and worse cognitive performance (Van Steenoven et al., 2016; Gomperts et al., 2016; Abdelnour et al., 2016; Lemstra et al., 2017), mainly in orientation and memory (Andersson et al., 2011; Tagawa et al., 2015).

Nevertheless, how AD-related pathology influences the neurodegenerative process in DLB is less studied. In AD, amyloid-β and tau-related pathologies are hypothesized to lead to neuronal injury (Hyman et al., 2012; Braak et al., 2006). DLB patients with concomitant AD-related pathology have shown a faster rate of brain atrophy over time measured with MRI, mainly in the medial temporal lobe, when compared with DLB patients without concomitant AD-related pathology (Nedelska et al., 2015; Sarro et al., 2016; Blanc et al., 2017; Nelson et al., 2009). This finding suggests that AD pathology may contribute to medial temporal lobe atrophy (MTA) in DLB (Elder et al., 2017; Sarro et al., 2016). Similarly, when amyloid is present the patterns of deposition and atrophy resembles that observed in AD (Shimada et al., 2013; Donaghy et al., 2015; Irwin and Hurtig, 2018; Mak et al., 2019ab) Understanding the underlying mechanisms of regional brain atrophy that could reflect distinct pathologies could have treatment implications. For example, a typical AD pattern of brain atrophy involving the medial temporal lobes and posterior cortices was associated with poorer response to acetylcholinesterase inhibitors in DLB patients (Graff-Radford et al., 2012). Also, amyloid or tau-targeted therapies might be effective in a subgroup of patients with DLB, i.e. it is important to improve our understanding in what degree AD pathologies may contribute towards personalized medicine approaches and improve differential diagnosis, disease prognosis, and treatment response in DLB.

Combining CSF biomarkers and structural MRI may inform about the mechanisms underlying regional atrophy, but few studies have been performed in DLB and results are inconsistent. A recent study reported an association between abnormal levels of CSF Aβ42 and MTA in DLB, as well as an association between abnormal levels of CSF total tau (T-tau) and global brain atrophy (van der Zande et al., 2018). By contrast, another study reported no differences between amyloid PET positive and negative DLB patients in hippocampal or gray matter volume (Donaghy et al., 2018). However, associations between regional atrophy and tau-related pathology have not been investigated yet. Importantly, in vitro studies have found cross-seeding of alpha-synuclein, amyloid and tau proteins (Spires-Jones et al., 2017), thus the combination of proteinopathies may have additional or even synergistic contributions to neurodegeneration. Hence, we aimed to explore this question by investigating the combined effect of CSF Aβ42, T-tau and p-tau on regional brain atrophy in the E-DLB cohort, a large multi-center study involving 19 centers from Europe (Oppedal et al., 2019). Our hypothesis was that DLB patients with abnormal levels of CSF Aβ42, T-tau and p-tau would have a higher level of brain atrophy, in particular, in the medial temporal lobes and posterior cortices, delineating the typical pattern of brain atrophy in AD (Oppedal et al., 2019).

2. Materials and methods

2.1. Participants population

A total of 86 DLB patients were selected from 6 centers of the E-DLB cohort. Inclusion criteria to enter the E-DLB cohort are reported in previous publications (Kramberger et al., 2017). For the current study, selection criteria were: 1) a diagnosis of probable DLB; 2) availability of CSF data; and 3) availability of MRI data. Detailed information about the centers that contributed to the current study is shown in Supplementary tables.

2.2. Diagnostic and clinical examination

Because the E-DLB cohort was assembled retrospectively, many patients had been diagnosed before 2017. Hence, The DLB diagnosis was made according to McKeith 2005 criteria (McKeith et al., 2005), but we were able to confirm the diagnosis of probable DLB according to McKeith 2017 criteria (McKeith et al., 2017) in 83 out of 86 patients. Diagnosis was made by the treating physician, a group of at least two expert clinicians, or a multidisciplinary team at a consensus diagnostic meeting on the basis of all available clinical and diagnostic test data as previously reported (Oppedal et al., 2019; Kramberger et al., 2017).

Clinicians interviewed both patients and caregivers, recorded demographic information as well as medical and drug history. All centers included a detailed medical history, aside from physical, neurological, and psychiatric examinations using standardized scales such as the motor subscale of the Unified Parkinson's Disease rating scale (Fahn et al., 1987) and the Neuropsychiatric Inventory (Cummings et al., 1994). Based on the clinical examination and/or the aforementioned scales the core diagnostic features fluctuating cognition, parkinsonism, and recurrent visual hallucinations were recorded as present or absent. Neuropsychological evaluation and complementary tests to rule out secondary causes of dementia (routine blood tests and brain imaging) were performed. 26 out of the total sample of 86 subjects had available DAT SPECT, 25 (96.15%) of which were abnormal.

2.3. Ethics

Local ethics committees at the individual centres approved the study. The patients gave their written consent to use the anonymised results of their clinical, instrumental and laboratory investigations for research purposes.

2.4. CSF procedures

CSF was obtained at all centers with the following procedures: 1) lumbar puncture at the L3-4 or L4-5 interspace; 2) collection in polypropylene tubes and centrifuged for 10 min at 4 °C; and 3) storage in aliquots of 0.5 mL at −80 °C or −70 °C until further analysis. Further details are summarized in Supplementary Table 1. CSF analyses were performed locally according to standard routines. INNOTEST enzyme-linked immunosorbent assays (ELISA) were used to analyze T-tau and p-tau (missing for 1 patient) in all samples and Aβ42 in 80 samples (Fujirebio, Ghent, Belgium). The remaining 6 samples were analyzed for Aβ42 using ELISA kits from Biosource Europe S.A. CSF values were dichotomized as normal or pathological based on well-established center-specific cut-off values for each biomarker as previously described (Abdelnour et al., 2016) (Supplementary Table A.1).

2.5. MRI analysis

Different neuroimaging acquisition protocols and MRI scanners were used as detailed in Supplementary Table A.2. The interval between MRI and CSF collection ranged from 0 to 3 months in the majority of the cases (73 out 86, which corresponds to 84.88%). In 13 patients the interval ranged from 3 to 12 moths (15.12%). We used visual rating of MRI scans, which is more feasible for clinical use than automated analysis, and is not influenced by between-center differences in acquisition protocols and MRI scanners. Ratings of all scans were performed by one expert radiologist (L. C.) as previously described (Ferreira et al., 2017), who has excellent intra-rater reliability - weighted κ of 0.94 and 0.89 for MTA in left and right hemispheres correspondingly, 0.88 for PA, and 0.83 for GCA-F in 120 random cases-(Ferreira et al., 2017) blinded to clinical data. T1-weigthed images were used to investigate regional brain atrophy by using three visual rating scales: the medial temporal lobe atrophy scale (MTA) (Scheltens et al., 1992), the posterior atrophy scale (PA) (Koedam et al., 2011) and the global cortical atrophy scale-frontal subscale (GCA-F) (Ferreira et al., 2016). Detailed information regarding the visual rating scales is provided elsewhere (Ferreira et al., 2017). The visual rating scores were dichotomized into normal and abnormal values in accordance with previously proposed cut-offs (Ferreira et al., 2015)

2.6. Statistics

The statistical analyses were done using R (www.R-project.org) version 3.2.4 and IBM SPSS version 26. Descriptive results are shown as mean ± SD for normally distributed continuous variables, and number and percentage for categorical variables.

The aim of this study was to investigate the combined effect of Aβ42, T-tau and p-tau (predictors) on regional brain atrophy as measured with visual rating scales (outcome variables). All these measures are dichotomous (0 normal, 1 abnormal). We also wanted to model the effects of age, sex, education and disease duration to investigate their possible added effect to the association between CSF biomarkers and regional brain atrophy. Age, education and disease duration are continuous variables while sex is dichotomous (0 males 1 females). Further, our interest was to investigate the predictive power of all these variables in combination as predictors of regional brain atrophy, rather than investigating their partial effects. Random forest (classification) (Breiman, 2001) was thus chosen given our aim, the nature of the variables, the number of predictors and the sample size. Random forest is an ensemble method in machine learning that involves growing of multiple decision trees via bootstrap aggregation (bagging). Each tree predicts a classification independently and votes for the corresponding class. The best model for each outcome variable is chosen from the majority of votes (Machado et al., 2018). Importantly, contrarily to other predictive methods such as multiple linear or logistic regression that investigate partial effects (competition among predictors in the prediction of the outcome), random forest investigates combined effects (the predictors do not compete with each other but "cooperate" in the prediction of the outcome) (Machado et al., 2018) Combined effects are closer to what we hypothesized in this study, i.e., amyloid-β (CSF Aβ42) and tau-related (CSF p-tau) pathologies have a synergistic deleterious effect on brain integrity. When CSF Aβ42 and CSF p-tau as predictors show a contribution to the prediction of brain atrophy, we can conclude that both pathologies have a combined effect on brain integrity, which may reflect their synergy at the pathological level (i.e. the "cooperation" between Aβ42 and CSF p-tau contributes to the prediction of brain atrophy). Further, random forest performs very similarly to other machine learning algorithms (Machado et al., 2018) but it was preferred in our current study due to the nature of our variables. We performed three random forest models: one for each atrophy scale (MTA, PA, and GCA-F) as the outcome variable. The random forest models were comprised of 5000 trees, providing an accurate estimation of the variables importance without introducing too much noise in the models due to the addition of redundant trees. Each of the trees was trained on randomly picked 70% of the data and subsequently tested on the unseen 30% of the data. Classification models (normal *vs.* abnormal) (Liaw and Wiener, 2002) were conducted, accounting for the fact that the outcome variable may present with an unbalanced amount of cases in its two levels (e.g. normal MTA n = 53, abnormal MTA n = 34). The classification error is reported as a measure of goodness of the model (out-of-the-bag estimated error rate, OOB-EER) (Breiman, 2001). When outcome variables are dichotomous, as it is our case, the error by chance is 50%. Therefore, a classification below 50% is better than chance, with values closest to 0% denoting better classification performance, hence good reliability of the model. We also report the importance (*Imp*) of the predictors as a measure of their contribution towards the prediction of the outcome variable (regional brain atrophy). Higher *Imp* values denote stronger contribution to the prediction. The random forest results were further complemented with the Pearson correlation coefficient to easily represent the magnitude and direction of the association between variables (bivariate association). P-values of Pearson correlation are reported for completeness of information.

3. Results

3.1. Sample features

Clinical and demographic features of the sample are reported in Table 1. Of the 86 patients, the number of patients with pathological CSF values is 28 (32.56%) for Aβ42, 17 (19.77%) for Total-Tau and 24 (27.91%) for p-Tau. The number of patients with abnormal scores in the visual rating scales was: MTA: 33 (38.37%), GCA-F: 34 (39.53%) and PA: 45 (52.33%). Fig. 1 shows 3 examples of different combinations for CSF Aβ42, CSF p-Tau CSF and the visual rating scales.

Table 1
Demographic and clinical data of the participants.

Features	Mean (SD)	Range
Age at diagnosis	69.36 (8.85)	49–88
Sex: Male N (%)	49 (56.98)	
Years of education	11.24 (4.08)	5–22
Disease duration (years)	4.04 (3.10)	0.5–14
MMSE	24.85 (3.72)	15–30
Parkinsonism (%)	82.6 (N = 71)	
Visual hallucinations (%)	58.1 (N = 50)	
Fluctuating cognition (%)	75.6 (N = 65)	

N: number. MMSE: Minimental State Examination.

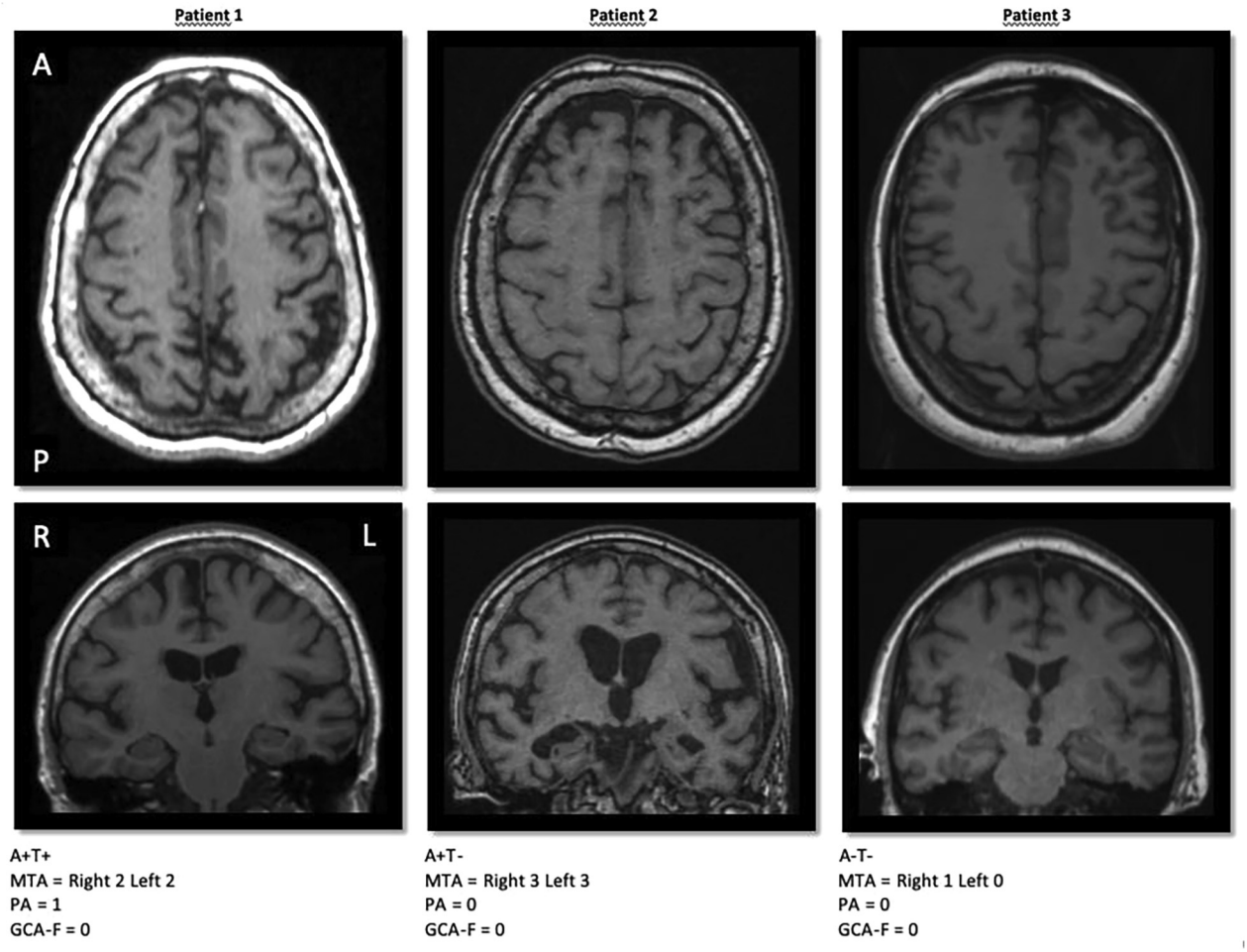

Fig. 1. Normal and pathological CSF values of Aβ42 and p-Tau combined with visual rating scales. CSF levels of Aβ42 and p-Tau were dichotomized according the cut-offs of each center into normal or pathological values. MTA, PA and GCA-F visual rating scales were used to measure regional atrophy based on T1-weigthed images. A+ = pathological CSF Aβ42; A− = normal CSF Aβ42; T+ = pathological CSF p-Tau; T− = normal CSF p-Tau; MTA = medial temporal atrophy scale; PA = posterior atrophy scale; GCA-F = global cortical atrophy scale – frontal subscale; A = anterior part of the brain; P = posterior part of the brain; R = right; L = left.

3.2. Association between AD CSF biomarkers and visual rating scores measured with MRI

The distribution of abnormal scores in the visual rating scales in relation to normal or pathological CSF Aβ42, T-tau and p-tau is presented in Table 2.

Classification performance in the three random forest models was better than chance: MTA, OOB-EER = 32.56%; PA, OOB-EER = 44.83%, GCA-F, OOB-EER = 24.14% (Table 3). The classification error for normal MTA was 24.52% and for abnormal MTA it was 45.45%. The classification error for normal PA was 46.34% and for abnormal PA it was 43.48%. The classification error for normal GCA-F

Table 2
Distribution of abnormal visual rating scores between normal and pathological AD CSF biomarkers groups.

	CSF Aβ42		CSF T-tau		CSF p-tau	
Visual rating scales	Normal	Abnormal	Normal	Abnormal	Normal	Abnormal
Abnormal MTA N (%)	18 (54.55)	15 (45.45)	28 (84.85)	5 (15.15)	26 (78.79)	7 (21.21)
Age (mean and SD)	69.78 (8.37)	73.93 (6.68)	70.86 (8.01)	76.20 (5.07)	70.27 (8.13)	76.86 (3.34)
Sex (Male N and %)	11 (61.11)	6 (40)	18 (64.29)	2 (40)	17 (65.38)	3 (42.86)
Disease duration (mean and SD)	3.64 (2.91)	2.43 (2.35)	3.11 (2.80)	3.00 (2.35)	2.87 (2.54)	3.93 (3.32)
Abnormal PA N (%)	25 (55.56)	20 (44.44)	33 (73.33)	12 (26.67)	29 (64.44)	16 (35.56)
Age (mean and SD)	67.24 (9.40)	75.70 (6.78)	70.24 (8.22)	73.08 (11.88)	69.17 (8.07)	74.31 (10.62)
Sex (Male N and %)	15 (60)	12 (60)	20 (60.61)	7 (58.33)	18 (62.07)	9 (56.25)
Disease duration (mean and SD)	3.98 (2.69)	2.48 (1.57)	3.26 (2.24)	3.46 (2.78)	2.91 (1.91)	4.03 (2.96)
Abnormal GCA-F N (%)	19 (55.88)	15 (44.12)	27 (79.41)	7 (20.59)	20 (58.82)	14 (41.18)
Age (mean and SD)	70.68 (9)	75.93 (7.06)	71.30 (8.57)	79.57 (6.05)	70.95 (8.57)	75.93 (8.36)
Sex (Male N and %)	14 (73.68)	11 (73.33)	20 (74.07)	5 (71.43)	15 (75)	10 (71.43)
Disease duration (mean and SD)	4.61 (3.08)	2.77 (2.15)	3.63 (2.74)	4.43 (3.31)	3.18 (2.20)	4.68 (3.44)

N: number. CSF: cerebrospinal fluid. Aβ42: Amyloid-β_{42}. T tau: Total tau. P tau: phosphorylated tau at threonine 181. MTA: medial temporal lobe atrophy. PA: posterior atrophy. GCA-F: global cortical atrophy scale-frontal subscale.

Table 3
Association between AD CSF biomarkers and visual rating scales (random forest models).

Visual rating scales	Variables contribution	Pearson correlation	P value
MTA	Overall model: OOB-EER = 32.56%		
	- Classification error normal MTA = 24.53%		
	- Classification error abnormal MTA = 45.45%		
	Predictors retained in the model:	−0.244	0.024
	Disease duration, Imp = 63.53	0.217	0.045
	CSF Aβ42, Imp = 41.71	0.207	0.056
	Age, Imp = 36.30		
PA	Overall model: OOB-EER = 44.83%		
	- Classification error normal PA = 46.34%		
	- Classification error abnormal PA = 43.48%		
	Predictors retained in the model:		0.072
	Age, Imp = 24.27	0.195	0.126
	Education, Imp = 8.76	−0.166	0.100
	CSF p-tau, Imp = 8.23	0.179	0.013
	CSF Aβ42, Imp = 8.615	0.266	0.021
	Disease duration, Imp = 7.02	−0.248	
GCA-F	Overall model: OOB-EER = 24.14%		
	- Classification error normal GCA-F = 19.23%		
	- Classification error abnormal GCA-F = 31.43%		
	Predictors retained in the model:	0.270	0.012
	Sex, Imp = 54.20	−0.231	0.033
	Education, Imp = 52.18	0.334	0.002
	Age, Imp = 46.23		

N: number. CSF: cerebrospinal fluid. Aβ42: Amyloid-β_{42}. T tau: Total tau. P tau: phosphorylated tau at threonine 181. MTA: medial temporal lobe atrophy. PA: posterior atrophy. GCA-F: global cortical atrophy scale-frontal subscale.
OOB-EER: out-of-the-bag estimated error rate (below 50% denotes good classification performance). *Imp*: importance (the contribution of a given variable in the random forest, with higher values indicating stronger contribution to the prediction). Pearson correlation indicates the direction of the association.

scores was 19.23% while it was 31.43% for patients with abnormal values. Table 3 shows that the best predictors of MTA were disease duration, CSF Aβ42 and age, ordered by importance. We found a combined effect of CSF Aβ42 and CSF p-tau on PA. Age, education and disease duration also contributed to the prediction of PA. Finally, the best predictors of GCA-F were sex, education and age. AD CSF biomarkers did not contribute to the prediction of GCA-F. The same pattern of results was observed when adding the center as a predictor in the models (data not shown), thus suggesting that variability across-centers does not seem to affect our findings.

Pearson correlation coefficients show that abnormal scores in MTA were related to abnormal CSF Aβ42 levels, whereas abnormal values of PA were associated with both abnormal CSF Aβ42 and p-tau levels. Regarding the effect of age, sex, education and disease duration, abnormal scores in MTA were related to shorter disease duration and older age. Abnormal scores in PA were related to older age, less education and shorter disease duration. Abnormal scores in GCA-F were related to less education, male sex and older age (Table 3). Fig. 2 shows the correlation matrix between visual ratings and CSF biomarkers, as well as among all predictors in our random forest models (Breiman, 2001).

4. Discussion

We found that both amyloid-β and tau-related pathologies contribute in combination to atrophy in posterior brain cortex of probable DLB patients. In addition, amyloid β-related pathology was associated with atrophy in the medial temporal lobe. In contrast, atrophy in the frontal cortex was not associated with AD-related pathology, and no associations were found between regional brain atrophy and global unspecific neurodegeneration (CSF T-tau).

Our results are consistent with previous findings on the association between amyloid β-related pathology and atrophy in the medial temporal lobe in DLB (Sarro et al., 2016; van der Zande et al., 2018; Shimada et al., 2013; Mak et al., 2019b; Kantarci et al., 2012a, 2012b). Although MTA is less frequent in DLB when compared with AD (Shimada et al., 2013; Barber et al. Mar, 2000; Ballmaier et al., 2004), previous studies suggest that when MTA is present, it could reflect concomitant AD-related pathology (Sarro et al., 2016; van der Zande et al., 2018). Both MTA and abnormal CSF Aβ42 levels are associated with more rapid cognitive decline in DLB and Parkinson's disease (Howlett et al., 2015; Siderowf et al., 2010; Stav et al., 2016; Caspell-Garcia et al., 2017). An interesting result of our study s that the classification error was higher for the prediction of patients with abnormal MTA scores as compared with the prediction of patients with normal MTA scores. This suggests that while in the absence of amyloid-beta pathology is unlikely to find MTA in probable DLB, the presence of MTA is not always associated with amyloid-beta pathology. Other factors than pathological CSF levels of Aβ42 may be involved in MTA in DLB. Our study shows that older age and shorter disease duration are associated with abnormal MTA scores. Future studies should also consider other pathologies potentially contributing to MTA, such as TDP-43 or hippocampal sclerosis.

Furthermore, we found that posterior brain atrophy was associated with both amyloid-β and tau-related pathologies. We acknowledge however that this finding should be considered as preliminary given the high classification error in our model (still under the threshold of error by chance). PET biomarkers are needed to confirm the collocation of amyloid and tau-related pathologies and neurodegeneration in the posterior cortex in DLB. Sarro et al showed that amyloid-β deposition is associated with greater atrophy rates in the posterior cingulate gyrus

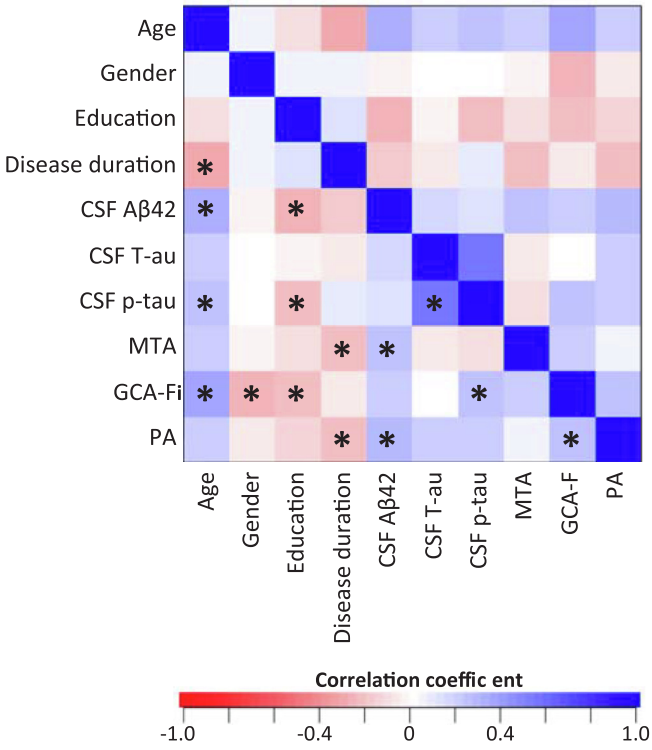

Fig. 2. Correlation matrix between visual ratings, CSF biomarkers, and predictors in the random forest models. Asterisk symbols (*) denote p-values < 0.05. MTA = medial temporal atrophy scale; PA = posterior atrophy scale; GCA-F = global cortical atrophy scale – frontal subscale.

and the occipital lobe in addition to the temporal lobe (Sarro et al., 2016). Investigation of tau-related pathology with ^{18}F-AV-1451 have found that DLB patients display increased uptake in the posterior and inferior temporoparietal, occipital (Kantarci et al., 2017) and parietal lobes (Smith et al., 2018). These findings indicate that tau-related pathology in DLB does not seem to follow AD Braak neurofibrillary tangle (NFT) distribution with the typical involvement of the medial temporal lobe (Braak and Braak, 1991), whereas amyloidosis presents with a diffuse cortical pattern similar to AD (Coughlin et al., 2019). Hence, the coexistence of DLB and AD pathologies could result in a distinctive pattern of regional brain atrophy in DLB. The novelty of our study is that we show that both amyloid-β and tau-related pathologies seem to be associated with level of atrophy in posterior cortex, while solely amyloid-β pathology appears to be related to atrophy in medial temporal lobes. This could be explained by a potential link between amyloid-β and tau-related pathologies in posterior brain areas, where tau pathology is primarily deposited in DLB (Kantarci et al., 2017).

Although both CSF T-tau and brain atrophy are considered markers of neurodegeneration, we did not find any association between the two. A possible explanation is that CSF T-tau is a marker of global unspecific neurodegeneration, while visual rating scales are markers of regional (local) neurodegeneration. AD studies show that the agreement between CSF T-tau and brain atrophy is limited (Alexopoulos et al., 2014). Moreover, we cannot exclude that this negative result is also explained by the small number of subjects with abnormal levels of CSF T-tau in our sample. Prior studies also observed that abnormal levels of CSF T-tau are less frequent than abnormal levels of CSF Aβ42 and p-tau in DLB patients (Mukaetova-Ladinska et al., 2010). Nevertheless, only one previous study analyzed CSF T-tau levels and regional brain atrophy in DLB, finding a correlation with posterior and global brain atrophy (van der Zande et al., 2018). Therefore, more studies are needed to determine the possible association between CSF T-tau -currently considered as a biomarker of neuronal damage- and brain atrophy in Lewy body dementia.

Similarly to what previously reported in AD, neither CSF Aβ42, T-tau nor p-tau were associated with atrophy in the frontal cortex in DLB (Ferreira et al., 2016). Previous research has demonstrated increased amyloid-β burden (Growdon et al., 2012) but not tau deposition (Kantarci et al., 2017) in frontal areas in patients with DLB. However, amyloid-β deposition in the frontal lobes has not been associated with grey matter atrophy in this region (Sarro et al., 2016). Frontal atrophy in DLB might be related to Lewy body pathology only, but more investigations are needed to elucidate the pathological mechanisms underlying the neurodegeneration of these areas.

Regarding the effect of age, sex, education and disease duration, we did not control for their effects but investigated to what extent they contribute to regional brain atrophy together with the CSF biomarkers. The decision to do so is because it is currently unknown whether these variables should be treated as confounding or contributing variables to regional brain atrophy in neurodegenerative disorders (Ferreira et al., 2020). For example, tau-related pathology is associated with increasing age, and it is currently unknown whether tau deposition in DLB is related to AD- or aging-, or both. Thus, including age, sex, education and disease duration in our models enabled us to investigate their combined effect together with the CSF biomarkers. We found that atrophy in medial temporal lobes and posterior cortices was associated with shorter disease duration and older age, whereas atrophy in the frontal cortex was associated with older age, male sex and less education. Further, our multivariate analyses exposed the effect of variables such as disease duration on the integrity of the brain, which traditional bivariate correlations could not capture in our study. In fact, multivariate models can capture effects of relevant variables masked by the effect of third variables that cannot be captured in univariate or bivariate models, and that are artificially removed in models testing for partial effects (Machado et al., 2018). This is therefore a strength of our multivariate statistical approach using random forest classification models. Another interesting finding is that the Pearson correlation between CSF p-tau and PA was not significant while CSF p-tau contributed to the prediction of PA in the multivariate random forest model. This suggests that the effect of tau-related pathology on the posterior cortex is not direct and instead emerges in combination with the effect of amyloid-β pathology. Hence, this dissociation between the results from our random forest and correlation analysis supports the potential synergistic effect of amyloid-β and tau-related pathologies when it comes to predict brain atrophy in the posterior cortex. Since age, education and disease duration were contributing variables in the random forest for PA, this suggests that AD-concomitant pathology in DLB may have a stronger impact on the posterior cortex in patients with lower education and older age, hence perhaps accelerating disease progression (i.e. shorter disease duration).

This study has some limitations. Firstly, we discuss on the observed association between CSF biomarkers and regional brain atrophy but our analyses are cross-sectional and we cannot assume causality. Still, we believe our findings may inform on potential underlying mechanisms of neurodegeneration in DLB, which need to be substantiated in future longitudinal studies. Secondly, the E-DLB cohort was assembled retrospectively using common registered variables and procedures across the participating centers. However, all variables and procedures are standard for clinical practice across centers and we carefully inspected the combinability of the data in order to exclude non-harmonized measures and cases. All centers have extensive clinical experience in the diagnosis of neurodegenerative diseases and align with international consensus diagnostic criteria, which we believe has contributed to minimize potential differences among centers. Further, we followed two more strategies to minimize methodological differences across-centers. We used cut-offs that were established at their respective center to dichotomize CSF biomarker results into normal or abnormal values, which is preferred in multi-center studies instead of continuous values. Similarly, due to the variability in the MRI protocols across centers, we used visual rating scales to investigate brain atrophy instead of more fine-grained automated methods or quantitative measures. Nonetheless, the use of visual rating scales substantially increases the clinical applicability of our current findings (Ferreira et al., 2017, 2015, 2020). On another hand, and connected to the retrospective nature of the cohort, it is worth mentioning that the interval between MRI and CSF collection was long in 15.11% of cases (ranged from 3 to 12 moths). Thirdly, there is no reliable in vivo biomarker of Lewy body pathology at present for diagnosis or analysis of the contribution of this pathology towards the neurodegenerative process. Thus, the diagnosis of probable DLB was based on clinical grounds with its known limitations (Rizzo et al., 2018; Huang and Halliday, 2013), although around one third of the cohort had a dopamine transporter SPECT scan. These limitations will be overcome in the prospective stage of E-DLB – we are currently collecting harmonized longitudinal data across many centers in Europe (Oppedal et al., 2019). Finally, although random forest is able to handle multicollinearity to some degree, it might lead to an underestimation of the contribution of multicollinear variables. The association among the predictors of the random forest models can be appreciated in Fig. 2. Our study has some important strengths. The study is a multicenter effort, which makes the generalization of the findings plausible through different clinical centers. Also, the use of modern multivariate models allowed us to investigate the combined effect of CSF biomarkers for the first time, in contrast to previous reports which investigated partial effects and could not investigate CSF p-tau in linear regression models due to collinearity (van der Zande et al., 2018).

5. Conclusions

This study shows preliminary data on the potential combined effect of amyloid-β and tau-related pathologies on posterior brain cortices in patients with DLB. Future research should confirm our current findings with more fine-grained automated methods for brain atrophy and,

ideally, with amyloid and tau PET biomarkers in order to verify the potential collocation of these pathologies with neurodegeneration in posterior brain cortices. Likewise, future studies should also include alpha-synuclein biomarkers when available in order to advance our current understanding of the neurodegeneration process in DLB.

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CRediT authorship contribution statement

Carla Abdelnour: Data curation, Formal analysis, Investigation, Methodology, Resources, Validation, Visualization, Writing - original draft, Writing - review & editing. **Daniel Ferreira:** Data curation, Formal analysis, Methodology, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Ketil Oppedal:** Data curation, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - original draft, Writing - review & editing. **Lena Cavallin:** Data curation, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing - review & editing. **Olivier Bousiges:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Lars Olof Wahlund:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Jakub Hort:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Zuzana Nedelska:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Alessandro Padovani:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Andrea Pilotto:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Laura Bonanni:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Milica G. Kramberger:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Mercè Boada:** Data curation, Investigation, Resources, Validation, Visualization, Writing - review & editing. **Eric Westman:** Conceptualization, Resources, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Javier Pagonabarraga:** Validation, Visualization, Writing - review & editing. **Jaime Kulisevsky:** Validation, Visualization, Writing - review & editing. **Frédéric Blanc:** Data curation, Investigation, Resources, Supervision, Validation, Visualization, Writing - review & editing. **Dag Aarsland:** Conceptualization, Project administration, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing.

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Appendix A. Supplementary data

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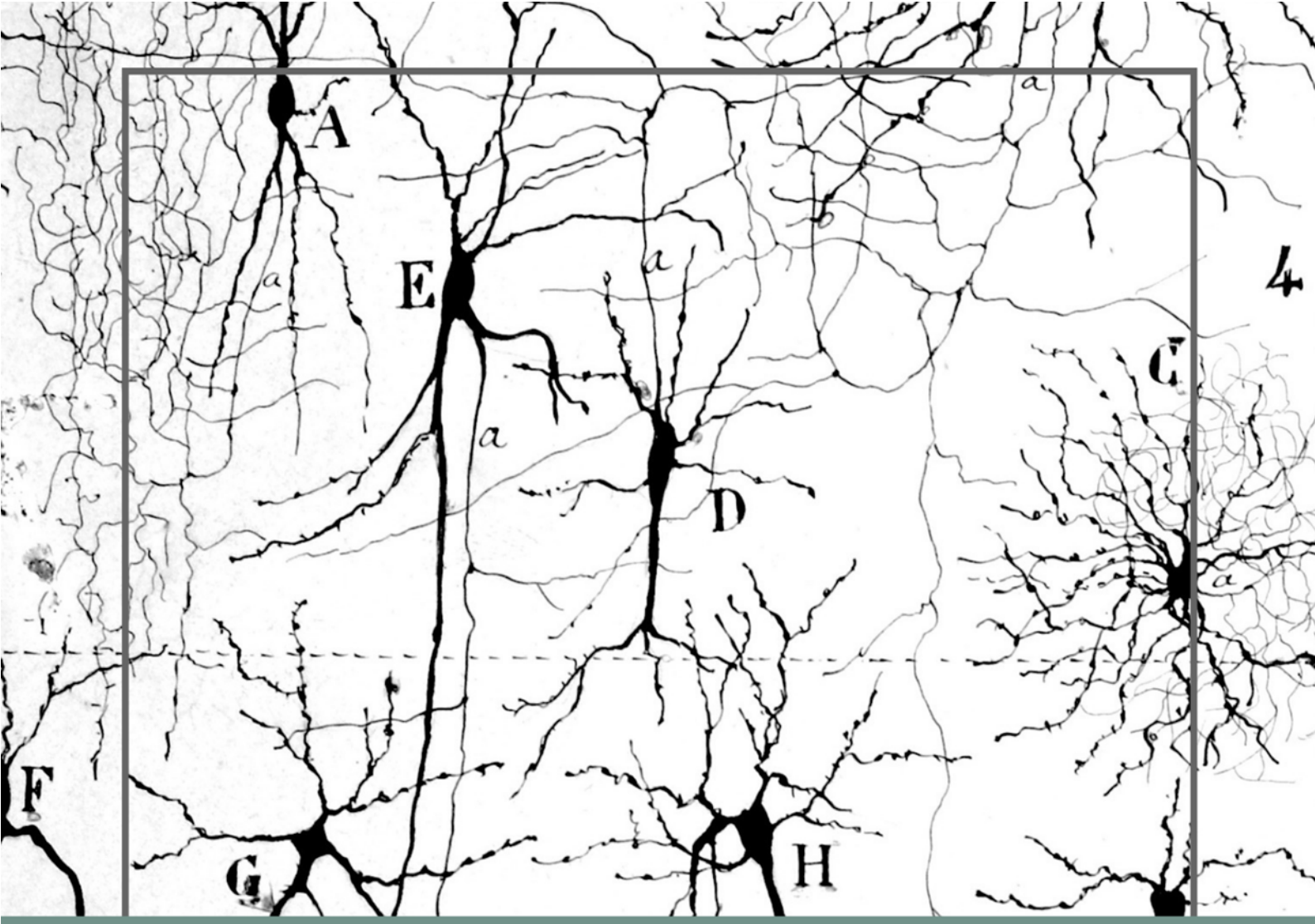

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