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APOLIPOPROTEIN E AND CHOLINERGIC ENZYMES: THE INTERFACE IN DEMENTIA

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Apolipoprotein E and Cholinergic enzymes: The interface in Dementia

THESIS FOR DOCTORAL DEGREE (Ph.D.)

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Dedicated to the dementia patients all over the world…

A special dedication to my little love Adithi Surendran ☺

"எது நடந்ததோ, அது நன்றாகவே நடந்தது
எது நடக்கிறதோ, அது நன்றாகவே நடக்கிறது
எது நடக்க இருக்கிறதோ, அதுவும் நன்றாகவே நடக்கும்
உன்னுடையதை எதை இழந்தாய், எதற்காக நீ அழுகிறாய்?
எதை நீ கொண்டுவந்தாய், அதை நீ இழப்பதற்கு?
எதை நீ படைத்திருந்தாய், அது வீணாவதற்கு?
எதை நீ எடுத்துக் கொண்டாயோ, அது இங்கிருந்தே எடுக்கப்பட்டது,
எதை கொடுத்தாயோ, அது இங்கேயே கொடுக்கப்பட்டது.
எது இன்று உன்னுடையதோ, அது நாளை மற்றொருவருடையதாகிறது,
மற்றொருநாள், அது வேறொருவருடையதாகும்.
இதுவே உலக நியதியும் எனது படைப்பின் சாராம்சமாகும்"

- பகவான் ஸ்ரீ கிருஷ்ணர்

ABSTRACT

The main confirmed genetic risk factor for sporadic form of Alzheimer's disease (AD) is apolipoprotein E (ApoE) ε4 allele (APOE4). ApoE protein aids in receptor ligand mediated transport, distribution and metabolism of fatty acids and cholesterol. However, the molecular mechanism of its contribution to the pathological events in AD is still highly obscure. Some reports implicate ApoE in amyloid-beta (Aβ) deposition in the brain, one of the main hallmarks of AD. But there are no consensuses about how ApoE causes the accumulation of Aβ deposits. The main neuronal network that becomes severely affected in the course of AD and Lewy body dementia disorders (LBD) is the central cholinergic system, and the main available therapeutic options are cholinesterase inhibitors which prevents the degradation of cholinergic neurotransmitter, acetylcholine (ACh). However, it is unclear, why these particular neuronal populations are selectively affected in AD and LBD. In addition to the classical view of the cholinergic neurotransmission, extrasynaptic cholinergic signaling also plays a crucial role in the regulation of immune system. Although, it is not clear how ACh can reach and act on such non-neuronal cell population in the brain or in the peripheral organs.

This thesis aimed to provide some insights in these unresolved issues. We found that APOE4 gene dose-dependently was associated with the highest ApoE protein expression in cerebrospinal fluid (CSF) of dementia patients (AD and DLB). This finding was observed in two independent study cohorts. Correlation analyses between the CSF ApoE protein and clinical measures (various cognitive tests), paraclinical measures (cerebral glucose metabolism and Aβ load by positron emission tomography) and AD biomarkers (CSF Aβ and tau, etc) suggested that high levels of ApoE protein may act as one of the driving forces of the pathological events in AD and DLB. This thesis also examined a postulated genetic interaction between APOE4 and a certain genetic variant of the ACh-degrading enzyme, butyrylcholinesterase (BuChE), namely BCHE-K allele. This directed genetic analysis was then to some extent scrutinized at their protein expression levels. The findings indicated that in the absence of APOE4, BCHE-K was associated with the lowest CSF ApoE protein, and the slowest annual cognitive decline, suggestive of some protective effects by BCHE-K. In the presence of APOE4, this protective effect of BCHE-K was either insufficient or rather increased the negative impact of APOE4 on both CSF ApoE levels and the rate of annual cognitive decline in patients.

Given that astrocytes, one of the key players of the immune system in the central nervous system are the main sources of ApoE and BuChE, this thesis also aimed at providing a

mechanistic understanding of the interplays between extrasynaptic cholinergic immune-regulatory signaling, Aβ, ApoE and BuChE.

In this context, our previous studies have provided evidence for APOE4 dependent molecular interaction between ApoE protein, Aβ and BuChE leading to formation of hyperactive BuChE-Aβ-ApoE complexes (BAβACs), which then accumulates in the brain, causing abnormal extrasynaptic ACh levels, astroglial dysfunction, altered Aβ clearance and aggregation in AD and DLB patients. Nonetheless, this hypothesis and the well-established cholinergic anti-inflammatory hypothesis faced a major dilemma, since ACh is a highly instable signaling molecule to be able to diffuse and act on cholinoceptive cells located far from the cholinergic synapses. This thesis provided a solution to this dilemma by providing for the first time, compelling evidence for the presence of ACh-synthesizing enzyme, choline acetyltransferase (ChAT) in human extracellular fluids, such as CSF and plasma. We showed that both lymphocytes and astrocytes might be the sources of soluble ChAT, as they were capable of acquiring cholinergic phenotype (expressing ChAT) on demand, and releasing ChAT into extracellular fluids. We showed that soluble ChAT was fully functional, and could maintain steady state ACh equilibrium in the presence of highly active ACh-degrading enzymes in CSF. Thus this thesis also provided for the first time evidence that astrocytes are cholinergic cells and hence might be an active part of the non-neuronal cholinergic network in the brain. In addition, the undertaking of this thesis led to development of a novel, sensitive integrated assay for sequential quantitative measurements of ChAT activity and protein levels in practically any biological fluid deemed to contain this enzyme.

Conclusions and future perspectives: This thesis provided important insights into the complex interplays that are ongoing *in vivo* in the course of two major dementia disorders (AD and DLB) between several key genetic and/or molecular factors and cellular networks. This thesis also provided a novel research tool, facilitating future studies for reviewing the above hypothesis in the field of dementia as well as other neuroinflammatory disease such as multiple sclerosis, which this thesis has pointed out.

LIST OF SCIENTIFIC PAPERS

I. **Vijayaraghavan, S.,** W. Maetzler, M. Reimold, C. U. Lithner, I. Liepelt-Scarfone, D. Berg and T. Darreh-Shori (2014). "**High apolipoprotein E in cerebrospinal fluid of patients with Lewy body disorders is associated with dementia**." *Alzheimer's & Dementia* **10**(5): 530-540. e531.

II. **Vijayaraghavan, S.,** A. Karami, S. Aeinehband, H. Behbahani, A. Grandien, B. Nilsson, K. N. Ekdahl, R. Lindblom, F. Piehl and T. Darreh-Shori (2013). "**Regulated extracellular choline acetyltransferase activity-the plausible missing link of the distant action of acetylcholine in the cholinergic anti-inflammatory pathway**." *PloS one* **8**(6): e65936

III. **Vijayaraghavan, S.,** T. Darreh-Shori, A. Rongve, G. Berge, S. B. Sando, L. R. White, B. H. Auestad, A. Witoelar, O. A. Andreassen, I. D. Ulstein and D. Aarsland (2016). "**Association of Butyrylcholinesterase-K Allele and Apolipoprotein E varepsilon4 Allele with Cognitive Decline in Dementia with Lewy Bodies and Alzheimer's Disease**." *J Alzheimers Dis* **50**(2): 567-576.

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LIST OF SCIENTIFIC PAPERS NOT INCLUDED IN THE THESIS

I. Darreh-Shori T, **Vijayaraghavan S**, Aeinehband S, Piehl F, Lindblom RP, Nilsson B, Ekdahl KN, Långström B, Almkvist O, Nordberg A (2013) **Functional variability in butyrylcholinesterase activity regulates intrathecal cytokine and astroglial biomarker profiles in patients with Alzheimer's disease**. *Neurobiology of aging* **34**, 2465-2481.

II. Malmsten L, **Vijayaraghavan S**, Hovatta O, Marutle A, Darreh‐Shori T (2014) **Fibrillar β‐amyloid 1‐42 alters cytokine secretion, cholinergic signalling and neuronal differentiation.** *Journal of cellular and molecular medicine* **18**, 1874-1888.

III. Karami A, Eyjolfsdottir H, **Vijayaraghavan S**, Lind G, Almqvist P, Kadir A, Linderoth B, Andreasen N, Blennow K, Wall A, Westman E, Ferreira D, Kristoffersen Wiberg M, Wahlund LO, Seiger A, Nordberg A, Wahlberg L, Darreh-Shori T, Eriksdotter M (2015) **Changes in CSF cholinergic biomarkers in response to cell therapy with NGF in patients with Alzheimer's disease.** *Alzheimers Dement* **11**, 1316-1328.

IV. Lindblom RP, Strom M, Heinig M, Al Nimer F, Aeinehband S, Berg A, Dominguez CA, **Vijayaraghavan S**, Zhang XM, Harnesk K, Zelano J, Hubner N, Cullheim S, Darreh-Shori T, Diez M, Piehl F (2014) **Unbiased expression mapping identifies a link between the complement and cholinergic systems in the rat central nervous system.** *J Immunol* **192**, 1138-1153.

V. Aeinehband S, Lindblom RP, Al Nimer F, **Vijayaraghavan S**, Sandholm K, Khademi M, Olsson T, Nilsson B, Ekdahl KN, Darreh-Shori T, Piehl F (2015) **Complement component C3 and butyrylcholinesterase activity are associated with neurodegeneration and clinical disability in multiple sclerosis.** *PLoS One* **10**, e0122048.

VI. Tajeddinn W, Persson T, Calvo-Garrido J, Seed Ahmed M, Maioli S, **Vijayaraghavan S**, Kazokoglu MS, Parrado-Fernandez C, Yoshitake T, Kehr J, Francis P, Winblad B, Hoglund K, Cedazo-Minguez A, Aarsland D (2016) **Pharmacological Modulations of the Serotonergic System in a Cell-Model of Familial Alzheimer's Disease.** *J Alzheimers Dis* **53**, 349-361.

CONTENTS

LIST OF ABBREVIATIONS

AD	Alzheimer's disease
AADC	Amino acid decarboxylase
ACh	Acetylcholine
AChE	Acetylcholinesterase
AChE-E	Acetylcholinesterase erythrocytic variant
AChE-R	Acetylcholinesterase readthrough variant
AChE-S	Acetylcholinesterase synaptic variant
AICD	APP intracellular domain
APOE4	Apolipoprotein E ε4 genotype
APP	Amyloid precursor protein
ASL	Arterial spin labeling
ATC	Acetylthiocholine
Aβ	Amyloid beta
BAβACs	BuChE-Aβ-ApoE complexes
BuChE	Butyrylcholinesterase
BTC	Butyrylthiocholine
CAIDE	Cardiovascular risk factors, aging and dementia
ChAT	Choline acetyltransferase
ChE	Cholinesterases
ChEIs	Cholinesterase inhibitors
CT	Computed tomography
DAT	Dopamine transporter
DLB	Dementia with Lewy bodies
DNA	Deoxyribonucleic acid
DSM	Diagnostic and statistical manual for mental disorders
DTBZ	Dihydrotetrabenzine
EDSS	Expanded disability status scale
EOAD	Early-onset Alzheimer's disease
FAD	Familial Alzheimer's disease
^{18}F-FDG	^{18}F-fluorodeoxyglucose
fMRI	Functional MRI
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GPI	Glycophosphoinositol

LOAD	Late-onset Alzheimer's disease
MMSE	Mini-mental state examination
MRI	Magnetic resonance imaging
MS	Multiple sclerosis
NINCDS-ADRDA	National Institute of neurological and communicative disorders and stroke-Alzheimer's disease and related disorders association
OND	Other non-inflammatory neurological conditions
PD	Parkinson's disease
PDD	Parkinson's disease with dementia
PET	Positron emission tomography
PIB	Pittsburgh compound B
PSEN	Presenilin
RNA	Ribonucleic acid
sAPP	Soluble amyloid precursor protein
SPECT	Single-photon emission computed tomography
TNF-α	Tumor necrosis factor-α
UPDRS	Unified Parkinson's disease rating scale
VAChT	Vesicular acetylcholine transporter
VMAT	Vesicular monoamine transporter

1 INTRODUCTION

1.1 HISTORY OF DEMENTIA

Dementia is an umbrella term that encompasses various memory disorders and mental disabilities, caused by the pathophysiological changes in the brain. Most of the dementia disorders are closely associated with age. Recorded evidence of dementia dates back 5000 – 6000 years. During the period, 3000-1500 BC, dementia had been described as Cittanasa (Citta means mind, nasa means loss of) in Ayurveda, an ancient Indian medical system which is still in practice. Ayurveda also has records of the herbal medications available for the treatment of dementia [1], however these need to be re-evaluated for their efficacy, using the modern medical techniques. Ancient Egyptians, around 2000 BC, believed that aging could be accompanied by memory disorders [2]. The Greek and Roman philosophers and physicians during 7^{th} century BC had also described the association of dementia with age [3]. In the modern age, Philippe Pinel (1745-1826), the founder of modern Psychiatry, gave a modern description of dementia [2].

1.2 ALZHEIMER'S DISEASE

Alzheimer's disease (AD) is a progressive neurodegenerative disorder leading to severe disruption in normal brain structure and function [4]. It accounts for 60-80% of the all dementia cases and has become the sixth leading cause of death in the United States. According to an Alzheimer's association report, by 2050, for every 33 seconds there will be a new individual developing AD in United States [5]. The first case of AD was described in 1906 by Dr. Alois Alzheimer, a German physician [4]. Hence this disease has been named after him.

The clinical symptoms of the disease include loss of memory, confusion, sleep disturbances, behavioral changes, language problems accompanied by difficulty in thinking, logical reasoning, visuospatial functions, sleeping and walking [6]. The typical disease pathology includes the presence of amyloid beta (Aβ) plaques and neurofibrillary tangles consisting of Tau protein deposits in the brain [7]. AD is also characterized by neuroinflammation, neuronal loss, synaptic dysfunction, cholinergic deficits, atrophy and low glucose metabolism in the brain [8-11]. Sometimes, the AD patients may also contain Lewy bodies in their brain [12] . However, which symptom occurs first and which follows next is still unclear. AD is more prevalent in women than men [13].

There are two major types of AD such as Familial AD (FAD) and Sporadic AD. (1) Familial AD is an early onset AD (EOAD) which accounts for about 15% of the total AD cases where the patients carry the risk genes for the disease due to their family history. FAD or EOAD is most common in patient groups who are <65 years of age. Three genes are closely associated with FAD - (i) amyloid precursor protein (APP) gene, (ii) presenilin 1 (PSEN1) and (iii) presenilin 2 (PSEN2), [14, 15]. (2) Sporadic AD or late onset AD (LOAD) occurs in people who do not have a family history of AD and most of the sporadic AD cases occurs at an age group >65 years. So far only Apolipoprotein E ε4 genotype (APOE4) and other epigenetic causes has been found to be closely associated with sporadic AD [15, 16].

1.3 DEMENTIA WITH LEWY BODIES

Dementia with Lewy bodies (DLB) constitutes for about 10-25% of the total dementia cases worldwide (http://www.alz.org/dementia/dementia-with-Lewy-bodies-symptoms.asp). Although the presence of α-synuclein inclusions called Lewy bodies, in the brain was first discovered in early 1900s by Frederick H. Lewy, the presence of Lewy body-like pathology in a patient with AD was only first reported in 1976, by a Japanese psychiatrist and neuropathologist [17] and was later named as "Dementia with Lewy bodies".

The pathology of the disease includes the presence of α-synuclein inclusions called Lewy bodies in the brain along with amyloid plaques and neurofibrillary tangles leading to excessive neuronal loss in the brain stem nuclei, neocortical and limbic regions [18-20]. DLB patients experience more sleep related disturbances than AD patients [21]. They present co-occurring AD and Parkinsonism, with symptoms such as motor dysfunctions, delusions, visual hallucinations, loss of synapses, cholinergic deficits, dopaminergic deficits, brain atrophy, low brain glucose metabolism and neuroinflammation which often leads to misdiagnosis of DLB most often as AD [22, 23]. Unlike AD, DLB is more common in men than women [24]. Whether, the genetic variant APOE4 plays an important role in DLB as it does in AD is not well known.

1.4 PARKINSON'S DISEASE

The symptoms of Parkinson's disease (PD) were first reported by Dr. James Parkinson in 1817 in his essay on "shaking palsy" [25]. Later, this disease was named after him as Parkinson's disease. It is one of the common types of neurodegenerative disorder, which mainly affects the motor functions causing rigidity, bradykinesia, tremor, slowness of movement and difficulty in walking [26]. The non-motor symptoms, such as depression, sleep disturbances, visuospatial deficits, often follow in the later stage of the disease, leading

to PD with dementia (PDD) [26, 27]. The PD pathology in brain is often characterized by the depletion of dopaminergic neurons and presence of Lewy bodies, especially in the mid brain region [28]. However, studies show that PD and PDD could also be accompanied by cholinergic deficits, synaptic dysfunction, Aβ plaques, Tau depositions and neuroinflammation [29]. The mutations in genes such as SNCA, LRRK2, VPS35, PINK1, DJ-1 and Parkin have been found to be associated with PD pathology [30].

1.5 DEMENTIA DIAGNOSIS

A diagnosis of dementia is based on detailed comprehensive assessment of findings from clinical history, physical examination and neuropsychological evaluation. This is often combined with neuroimaging and cerebrospinal fluid biomarker investigations. Important dementia risks, including cardiovascular abnormalities risk factors and senescence can be quantified using the CAIDE scale [31]. Dementia can be diagnosed using consensus criteria, such as the Diagnostic and Statistical Manual for Mental Disorders (DSM 5) criteria [32-34] or International Classification of Diseases -10 criteria (ICD-10,) which classify mental and behavioral disorders [35]. Many studies have employed different criteria for the specific diagnosis of different types of dementia, including National Institute of Neurological and Communicative Disorders and Stroke-Alzheimer's Disease and Related Disorders Association (NINCDS-ADRDA) criteria for AD [36], revised consensus criteria from the DLB consortium [37] for DLB and guidelines from the Movement Disorder Society [38, 39] for PD dementia. Despite the availability of specific procedures for the differential diagnosis of AD, DLB, PD and PDD, the overlapping clinical and pathological characteristics of the diseases can lead to misdiagnosis. Relatively few reports have been published with a comparison of the features of AD, DLB and PDD in the same study [40-43].

1.5.1 Clinical assessment of dementia

The clinical assessment of dementia is mainly based on the medical history and evaluation of the cognitive ability of the patients with cognitive tests. Initial screening tests may be used such as the Mini-Mental State Examination (MMSE). More targeted testing may assess language, e.g. Boston-naming test; psychomotor speed, e. g. Trail Making test A and B; verbal memory, e.g. California Verbal Learning test; as well as complete neuropsychological batteries, e.g. the Halsted-Reitan battery. Assessment of depression is important since depression is a risk factor for subsequent dementia; and may be the presenting symptom of dementia, as well as act as a confounding factor in cognitive testing [44]. Unified Parkinson's

Table 1. Characteristic features of AD, DLB and PD

	AD	DLB	PD
Neuropsychiatric symptoms			
Visual hallucinations	+	+++	++
Delusions	++	+++	+
Depression	++	++	++
Apathy	++	++	+
	Hallucinations in late stages of disease	Persistent hallucinations early in course of disease	In association with anticholinergic dopaminergic drugs
Tremor	−	++	+++
Rigor	+	+++	+++
Bradykinesia	+	+++	+++
	Rare, usually mild in late stages	Similar severity as in PD, pronounced rigidity and bradykinesia	First manifestation of disease, initially often asymmetric
Fluctuation of cognition	+	+++	−
		Prominent, severe, early in the course	
Neuropsychology	Early impairment of declarative memory and retention	Early disturbances in attention, visuo-perceptive functions	Impaired executive functions
Neuroimaging			
Global brain atrophy	++	++	−
Medial temporal lobe atrophy	+++	+	−
Occipital hypoperfusion		+++	+
Impaired dopaminergic activity	−	+++	+++
Neuropathology and chemistry			
Senile plaque density	+++	++	−
Tangle density	+++	++	−
Subcortical LB	−	++	+++
Cortical LB	−	+++	+
Cholinergic deficit	++	+++	+
Dopaminergic deficit	−	++	+++
Genetics			
Overrepresentation APOE4	++	++	−

+++ Indicates typical manifestation of disease, ++ indicates usually present, + indicates present, − indicates absent

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Disease Rating Scale (UPDRS) and Hoehn and Yahr Staging are commonly used standardized instruments to assess the severity or stage of Parkinsonism in patients. **Table 1** shows a comprehensive note of the observed neuropsychiatric, neuropathological, neurochemical, neuroimaging and genetic differences between AD, DLB and PD patients

1.5.2 Pathophysiological markers in dementia

1.5.2.1 Cholinergic system and its markers

(i) Cholinergic versus Cholinoceptive markers

Cholinergic system is comprised of nerve cells, which use the neurotransmitter ACh for the transduction of action potentials both in the central and peripheral nervous system. These nerve cells are defined as cholinergic if they are capable of producing and releasing ACh. In contrast, all other cells, including cholinergic neurons could be cholinoceptive, if they express ACh receptors, which are activated by the ACh molecule. The enzyme, choline acetyltransferase (ChAT, EC 2.3.1.6), reversibly catalyzes the synthesis of ACh from its precursors molecules choline -- a natural amine found in various fatty acids in the lipid

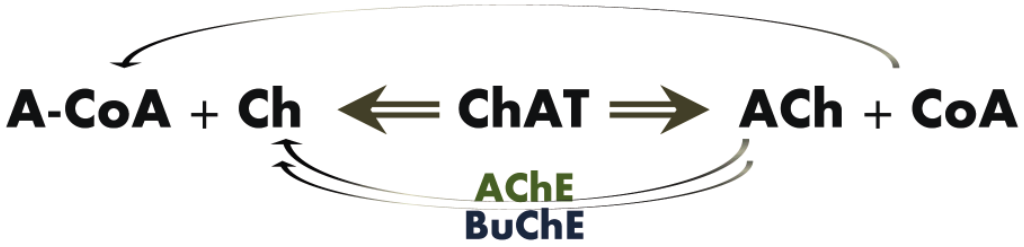

Figure 1. The chemical reaction involved in Acetylcholine (ACh) synthesis.
The synthesis of ACh from choline (Ch) and acetyl coenzyme A (Acetyl CoA) is catalyzed by the enzyme choline acetyltransferase. After its release, ACh is hydrolyzed by the cholinesterases into Ch. The source of Acetyl CoA is believed to be from the Citric acid cycle in mitochondria.

bilayer of the cell membrane, and acetyl coenzyme A (Acetyl CoA) -- a thioester used in metabolic reactions that acts as an acceptor and donor of acetyl groups [45]. The ACh produced in the nerve cells are packed inside small vesicles with the help of VAChT, which are then released into the synapses during an action potential. In the synapse, ACh binds to its receptors, namely nicotinic (nAChRs) and muscarinic (mAChRs) ACh receptors present in the adjacent cells. Following the dissociation from the receptors, ACh is readily hydrolyzed by the cholinesterases (ChE): AChE (EC 3.1.1.7) and butyrylcholinesterase (BuChE, EC 3.1.1.7) into choline and acetic acid (**Figure 1**). The choline from the ACh hydrolysis is

mostly recycled through active reuptake by a high affinity choline transporter into the cholinergic cells [46-48] (**Figure 2)**.

(ii) Choline acetyltransferase (ChAT)

The involvement of ChAT in ACh synthesis has been known since 1943 [49]. However, the extremely low amounts and instability of this enzyme in tissues, make it difficult to analyze its biochemical and structural functions. Normally ChAT exists in two different forms - soluble form and non-ionic membrane bound form in the cholinergic nerve terminals. The soluble ChAT contributes to 80-90% of the total enzyme activity, whereas the membrane bound ChAT contributes to 10-20% of the enzyme activity [50]. Different splice variants of ChAT such as M, R and N types have been identified from the coding gene region at the ‘‘cholinergic gene locus’’ in rodents and humans. All splice variants produce the same ChAT isoform in rodents. Whereas, in humans the M-type mRNA has the ability to generate both large (82 kDa) and small forms (69kDa) of ChAT and R-type and N-type ChAT mRNA generates a small form, which corresponds to the rodent ChAT [50]. Another classification of ChAT refers to a common type (cChAT) in the central nervous system (CNS) and a peripheral type (pChAT), the latter which is preferentially expressed by the peripheral neurons and is expected to have a Mw of 50 kDa [51]. Studies also report the presence of small 27kDa ChAT protein produced by another mRNA splice variant. This smaller variant appears to lack the catalytic activity, but it might have a regulatory role on the activity of full-length ChAT [52]. The different genetic variants of ChAT gene is also found to be associated with the cognitive decline in AD patients [53].

Several studies from the past have shown the existence of the ACh synthesizing enzyme, ChAT in the intracellular compartments. But in order to modulate immune functions, ACh must diffuse to a considerable distance from the cholinergic nerve terminals, resisting the action of two extremely efficient ACh-degrading enzymes, AChE and BuChE, which are abundant in the extracellular fluids such as plasma and CSF. In addition, the immuno-suppressive activity requires that ACh to be present at certain extrasynaptic levels to exert its putative role on immune cells by activating a7-nAChR ion-channels. Hence, a link is missing for the distant action of ACh in the cholinergic anti-inflammatory pathway. Considering the cholinergic hypothesis in dementia, the role of ChAT in AD, DLB, PD and PDD patients in maintaining the ACh levels in the brain and the interstitial fluids needs more clarification.

(iii) Acetylcholinesterase (AChE)

AChE encoded by a gene on chromosome 7 is the most predominant form of cholinesterase in the brain. It helps in terminating the synaptic neurotransmission by acting on the ACh molecule, which is hydrolyzed by them into choline and acetic acid. It is expressed in both cholinergic and non-cholinergic neurons in the central and peripheral nervous system. Additionally AChE is expressed in lower proportions by the cells and tissues in the brain and elsewhere such as in the meninges and glial cells [54]. Studies also show its presence in the red blood cell membranes [55]. Three different splice variants of AChE exist, namely the synaptic variant (AChE-S), the read-through variant (AChE-R) and the erythrocytic variant (AChE-E). The synaptic form constitutes the major species in the brain and mostly exists as a globular tetrameric form (G4). The AChE-R mostly exists as a monomeric (G1) form in the brain and the AChE-E exists as a glycophosphoinositol (GPI) anchored dimers (G2) [54].

The membrane bound AChE plays an important role in maintaining the ACh levels in the cholinergic synapses. High AChE activity in brain is considered as a typical characteristic of dementia, which causes an imbalance in ACh levels and, thereby, leads to cognitive symptoms in patients. Apart from the major role as ACh hydrolyzing enzyme, AChE also plays an important role in neurogenesis, cell adhesion, synaptogenesis, activation of dopamine neurons, promoting amyloid fiber assembly, hematopoiesis and thrombopoiesis [48]. The splice variants AChE-S and AChE-R are found to be associated with dementia and previous studies from our group have shown that AChE R/S ratio is reduced in untreated AD patients, and it's increase after treatment with cholinesterase inhibitors (ChEIs) constitutes a positive sign [56].

(*iv) Butyrylcholinesterase (BuChE)*

BuChE also known as pseudo cholinesterase is a serine hydrolase, and is responsible for the hydrolysis of ACh in the extracellular fluids. It acts as a co-regulator of cholinergic system along with AChE. In human brain the glial cells are the major source of this enzyme. However, it is also expressed in the neurons and endothelial cells in both central and peripheral nervous system. The BuChE also exists in three molecular forms such as G1, G2 and G4, which are all symmetrical, hydrophilic, and globular. The G1 form is predominant in the developing brain, whereas the G4 form is predominant in the mature brain [57].

The butyrylcholinesterase gene (BCHE) is located in the long q-arm of chromosome 3 and over 40 mutations have been identified in this gene. The BCHE-K variant is the most

common polymorphism of BCHE, which is resulted due to the substitution of an alanine residue at codon 539 to threonine (A539T). Other mutations of BCHE gene include the atypical, J, H, and silent variants. The distribution and activity of BuChE in CSF, brain and serum varies depending on the type of genetic variant present in an individual [57].

Recent studies have indicated a high level of BuChE protein and activity in the brain and CSF of dementia patients [57]. Evidences shows large amount of BuChE deposits in amyloid plaques and neurofibrillary tangles in dementia patients suggesting an important role of BuChE in dementia especially in AD [57, 58].

(v) Cholinergic hypothesis in AD

During senescence and dementia, degeneration of the cholinergic neurons occur in the cerebral cortex and hippocampus regions of the brain, which are responsible for cognitive functioning. This leads to a decrease in ACh mediated neurotransmission and ACh levels in the brain and periphery thereby causing cognitive deficit [59, 60].

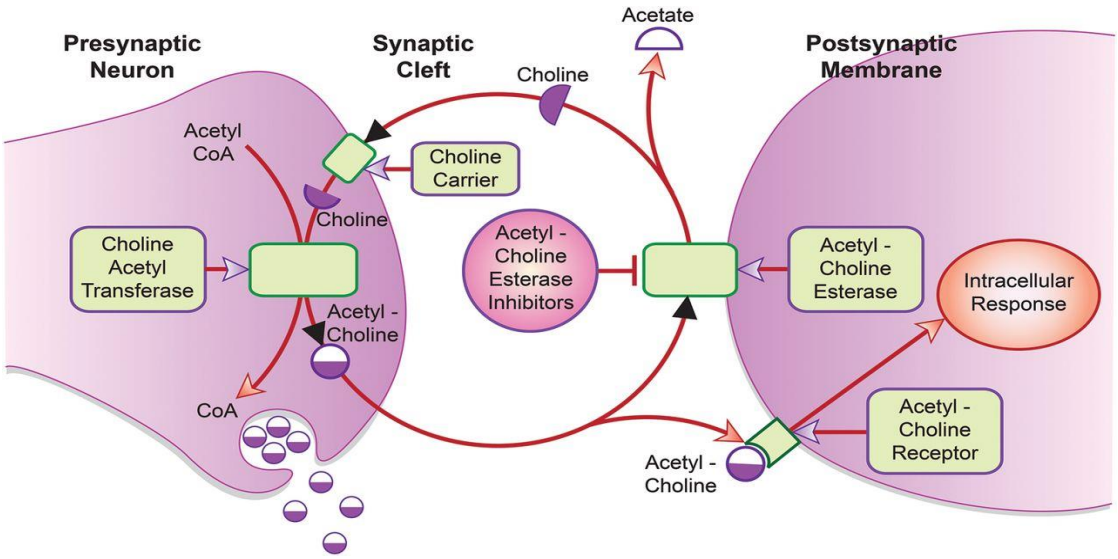

Figure 2. Cycle of acetylcholine. Acetylcholine is produced in the presynaptic neuron by the enzyme choline acetyltransferase from acetyl-coenzyme A and choline, and later released in the synaptic cleft where it binds to the acetylcholine receptor on the postsynaptic membrane, initiating an action potential and/or triggering an intracellular response. The enzyme acetylcholinesterase hydrolyses acetylcholine into acetate and choline in order to terminate synaptic transmission. Choline is transported into the presynaptic neuron by the choline carrier and serves as a substrate for the described production of acetylcholine. By inhibiting the enzyme acetylcholinesterase, the AChEIs prevent the breakdown of acetylcholine into acetate and choline, prolonging its duration of action.
Jeger, R.V.,European heart journal
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ACh through the cholinergic anti-inflammatory pathway, helps the nervous system to exert immuno-modulatory effects on the systemic immunity [61, 62]. This is coined, the cholinergic anti-inflammatory reflex or pathway (CAP). In this context, ACh putatively acts as a suppressor of inflammatory responses by lymphocytes by binding to the α7nAChRs present in these cells [63].

1.5.2.2 Apolipoprotein E (ApoE)

Human ApoE protein is a 299-amino acid glycoprotein with a molecular weight of 34kDa, which is expressed by several cell types in the central nervous system. ApoE is one of the major classes of lipoproteins in brain and is primarily expressed by astrocytes and microglia [64]. The highest production of ApoE in periphery occurs in the liver. ApoE plays a major role in mediating cholesterol metabolism and lipid transportation [64].

In human, APOE gene is located in the chromosome 19. There are three major alleles of APOE namely ε2, ε3 and ε4 expressing ApoE2, ApoE3 and ApoE4 isoproteins, respectively. The three isoforms of ApoE differ from each other at the amino acid residues 112-158 which contains either cysteine or arginine. ApoE3, the most common isoform contains Cys112, Arg158; APOE4 the next common isoform contains Arg112, Arg158 and APOE2 the least common isoform has Cys112 and Cys158. The single amino acid difference in these protein isoforms alters its ability to bind to receptors, ligands and other associated proteins such as Aβ **(Figure 3**[65]**).**

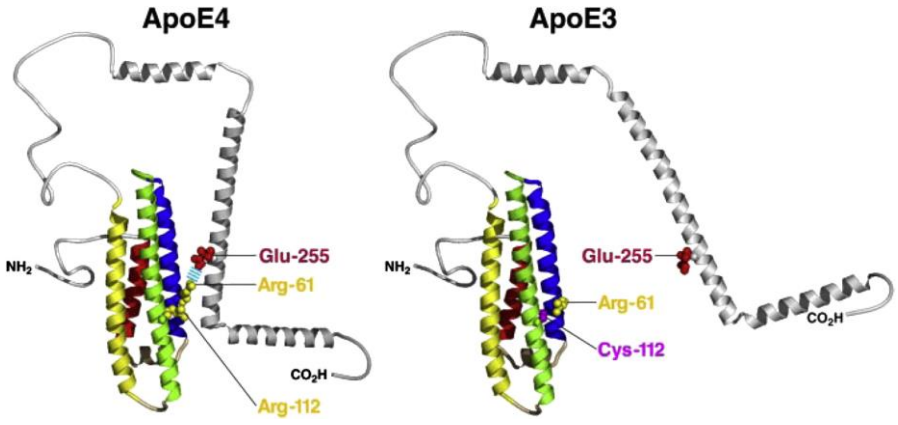

Figure 3. Structural difference between ApoE3 and ApoE4.
Zhong and Weisgraber,Trends in molecular science
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APOE3 is the most common type of allele and is present in more than half of the general population [66]. APOE4 genotype is considered as the major risk factor for AD, whereas

APOE2 genotype has been found to be a protective factor [67]. APOE4 may also act as a risk factor in other types of dementia such as DLB [68].

The level of ApoE in CSF varies with the specific type of APOE genotype. Previous studies from our group show that APOE4 is associated with high CSF ApoE levels in AD patients [69]. APOE4 may also trigger inflammatory cascades in the brain, which could have an impact on the pathogenesis in dementia patients [70].

ApoE binds to several receptors belonging to the low-density lipoprotein receptor (LDLR) family in an isoform dependent manner. ApoE and its receptors may play a major role in Aβ aggregation and clearance, which also varies with the different isoform of the ApoE protein. [71]. Recently, it has been shown that ApoE may also interact with Aβ and prevents its fibril formation in a concentration and isoform dependent manner [72]. Apart from this, ApoE and its receptors also seem to play a vital role in Tau phosphorylation, amyloid precursor protein (APP) processing, synaptic plasticity, brain atrophy, neuronal cell survival and integrity [68, 71].

1.5.2.3 Amyloid precursor protein (APP)

APP is an integral transmembrane protein and the APP gene is located in the chromosome 21 in humans. The cleavage of APP occurs in two ways, namely, amyloidogenic and non-amyloidogenic pathway. In the non-amyloidogenic pathway, APP is cleaved by the enzymes, ϒ-secretase and α-secretase. The latter cleaves APP at an epitope within the Aβ sequence in APP protein, excluding the production of Aβ peptides and producing a small p3 peptide, a soluble APPα (sAPPα), and APP intracellular domain (AICD) fragments. Whereas in the amyloidogenic pathway, APP is cleaved at by ϒ-secretase and β-secretase producing the Aβ peptide (an Aβ 40, 42 or 43), sAPPβ and an AICD fragment. Hence by definition, high levels of sAPPβ in the brain could indicate the prevalence of amyloidogenic pathway and hence more Aβ production which could aggregate and accumulate as Aβ plaques. However, the differences between the levels of sAPPα and sAPPβ in the CSF remains unclear, especially in sporadic AD [73]. Thereby, the amyloidogenic and non-amyloidogenic terminology might be misleading, in particular, since Aβ production is apparently a native biological process, since it occurs in all human subjects regardless of age or disease. Whilst “amyloidogenic” ascribes a general protein aggregation process that may occur for diverse proteins, such as prions, α-synuclein, huntingtin, serum amyloid A, etc. Thus usage of the neutral terms, α-secretase and β-secretase cleavage pathways, are best justified.

Nonetheless, sAPPα is associated with several protective functions in the brain such as promoting neurite outgrowth, synaptogenesis and cell adhesion, although the exact mechanisms behind its functions is not fully understood. sAPPβ lacks the neuroprotective effects when compared to sAPPα, but seems to be critically involved in the pruning of synapses during the development of both the central and peripheral neurons [73].

1.5.2.4 Amyloid β and Tau

APP, undergoing amyloidogenic (β-secretase cleavage) pathway produces Aβ fragments of varied length, such as Aβ 38, Aβ 40 and Aβ 42. Such Aβ deposition in the brain is considered as one of the hallmarks in AD pathogenesis. If present in excess (and/or under certain conditions), these peptides form fibrils and accumulate as plaques in the brain of patients with AD and other dementia such as DLB and PDD. Aβ 42, which is generally believed to be the most neurotoxic Aβ peptide, is known to be hydrophobic, and prone to aggregate much faster than Aβ 40 and Aβ 38. The typical AD pathology includes the presence of high Aβ aggregates in the brain and low Aβ42 in CSF. There are different molecular forms of Aβ, such as monomers, dimers, tetramers, oligomers, fibrils, plaques etc. However, which molecular form of Aβ is most toxic and acts as a key cause for AD or other dementia is unclear. There are no general correlations between cognition and/or clinical severity of the disease and the Aβ levels in CSF or the brain (PET-tracer data). For instance, MCI patients exhibit similar levels of Aβ deposits as AD patients, and ~30% of control subjects are Aβ-PET positive. Aβ might nonetheless be associated with AChE, BuChE, ApoE and inflammation in the brain and CSF thereby increasing the risk of dementia [74-76].

Neurofibrillary tangles are intracellular deposits of hyper-phosphorylated Tau protein in the brain, constituting another major hallmark of AD and the related dementias. Tau is a microtubule-associated protein, abundantly present in the central and peripheral nervous system. It helps in microtubule assembly and its stabilization in the brain. During dementia, hyper-phosphorylation of Tau alters the microtubule stability resulting in loss of synapses, tangle formation and neuronal death. Tau protein levels in CSF and brain are also associated with poor cognition in dementia patients [77].

1.5.3 Neuroimaging markers in dementia

Neuroimaging has become a very important diagnostic tool in dementia to investigate the structural, functional and pathological changes in the brain. Some of the commonly used neuroimaging biomarkers for dementia diagnosis are listed below.

1.5.3.1 Structural imaging

Computed tomography (CT) and magnetic resonance imaging (MRI) are commonly used for determining the structural changes in the whole brain and specific regions of the brain. The different modes in MRI give detailed information regarding the volumetric changes in the brain such as atrophy, micro-bleeds and grey and white matter changes. CT and MRI are used for primary clinical diagnosis of dementia due to cheaper cost and ease of use [78]. These techniques also helps to rule out the structural lesions in dementia [79].

1.5.3.2 Functional imaging

The alterations in the neuronal activity in the brain while performing cognitive tasks are characterized by altered blood flow in the brain, which is measured using functional MRI (fMRI). Resting state fMRI helps in measuring the functional changes in the resting-state networks of the brain. It also helps in measuring the functional changes in the brain of patients who are too impaired to perform any cognitive tasks. Thus, fMRI provides information regarding the functional changes in the brain due to neurodegeneration [78].

1.5.3.3 Glucose metabolism in brain

Positron emission tomography (PET) involves the injection of a radiolabelled compound into the body to bind with the target of interest. ^{18}F-fluorodeoxyglucose (^{18}F-FDG)-PET is used to measure the glucose metabolism in the brain. Dementia patients exhibit low glucose metabolism in the brain, which is associated with increased neurodegeneration [78].

1.5.3.4 Brain perfusion

Single-photon emission computed tomography (SPECT) involves the use of a radiolabelled ligand to study cerebral brain perfusion, which gives a measure of the regional blood flow in the brain and is used as an indicator of severity in dementia [78, 79]. Brain perfusion measured using arterial spin labeling (ASL) provides quantitative imaging of the cerebral blood flow using MRI equipment. This could help in the early characterization of AD [80]

1.5.3.5 Amyloid-beta deposition in the brain

The Aβ deposition in the brain is commonly measured with the help of ^{11}C-Pittsburgh compound B (PIB)-PET. The presence of high Aβ in the brain constitutes for the major pathological changes in AD and DLB patients and this pattern of Aβ deposition vary between different brain regions in different type of dementia. The ^{11}C-PIB when injected into the body binds putatively to fibrillar forms of Aβ deposits in the brain and thus this method of brain

imaging along with other imaging techniques such as CT, MRI and ^{18}F-FDG-PET might help in the differential diagnosis of dementias such as AD, DLB, PD and PDD [78, 81]. Apart from ^{11}C-PIB, there are also other PET tracers available for Aβ imaging, such as 18 F-flutemetamol, 18 F-florbetapir and 18 F-florbetaben. However, the diagnosis with PET imaging due to its higher costs, cannot be afforded for all dementia patients. **Figure 4** shows the amyloid imaging in the brain of AD, DLB, PD, PDD patients and aged healthy control [82].

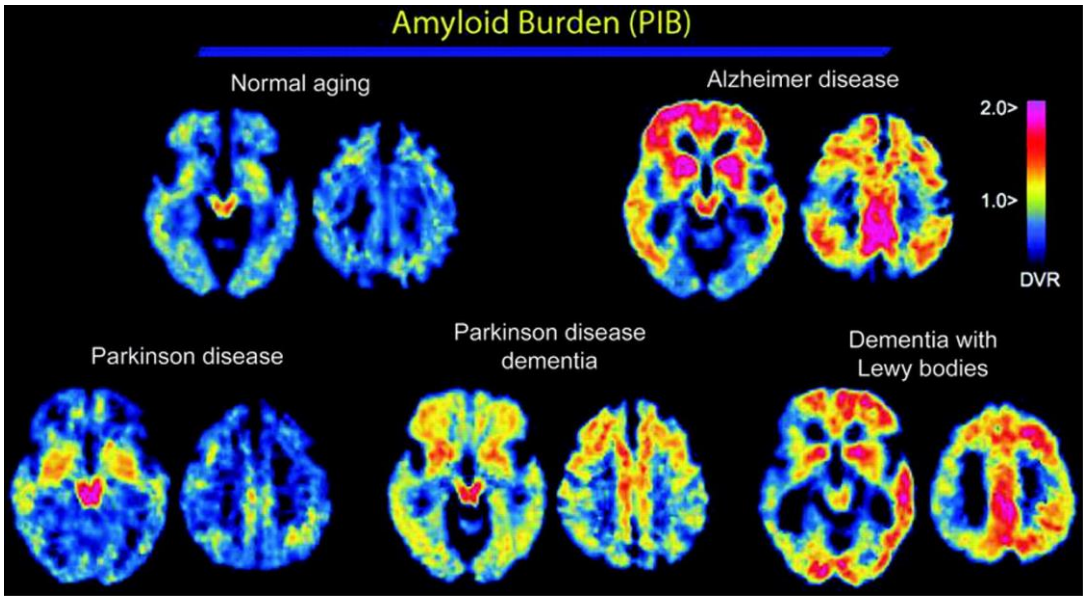

Figure 4. Amyloid imaging (PIB-PET) in the brain of normal aging individual, AD, PD, PDD and DLB patients. Images from a 75-year-old normal control (NC) (upper left), a 79-year-old patient with Alzheimer disease (AD) (Mini-Mental State Examination (MMSE) score 25; upper right), a 65-year-old patient with Parkinson disease (PD) (MMSE score 27; lower left), a 69-year-old patient with PD dementia (PDD) (MMSE score 25; lower middle), and a 71-year-old patient with dementia with Lewy bodies (DLB) (MMSE score 8; lower right) are displayed. Note that Pittsburgh Compound B (PiB) retention is qualitatively increased in AD, PDD, and DLB compared with NC and PD. Note also the variation in the regional distribution of amyloid across the AD, PDD, and DLB images. DVR=distribution volume ratio.
Gomperts, S., et al., Neurology
Reprinted with permission from Neurology.

1.5.3.6 Dopamine load in brain

The dopamine depletion in the brain results in movement disorders associated with the Parkinsonism observed in DLB, PD and PDD patients. The dopamine load in the brain can be visualized by measuring the Dopamine transporter (DAT), which is located presynaptically on the dopamine releasing terminals using DaTscan-SPECT. The vesicular monoamine transporter (VMAT) and amino acid decarboxylase (AADC) present in the dopamine

releasing neuronal terminals can be measured using ^{11}C- Dihydrotetrabenzine (DTBZ) and ^{18}F-dopa respectively. These three scans greatly help in studying the dopamine deficit in the brain and thereby also help in differentiating PD, PDD and DLB from healthy subjects and other kinds of dementias with high accuracy [81, 83].

1.5.3.7 Other neuroimaging markers

Recently there have been studies reporting the development of a radioactive PET tracer for quantifying the Tau protein in the brain, which is also a hallmark of AD and neurodegeneration in dementia [84]. Also the astroglial activity in brain, which is associated with neuroinflammation and neurodegeneration in dementia is studied in vivo using autoradiography techniques [85]. PET imaging of cholinergic markers such as acetylcholinesterase (AChE) and SPECT imaging for α4β2nAChRs and vesicular acetylcholine transporter (VAChT) have also been developed for the better understanding of cholinergic system in dementia patients [86].

2 AIMS

2.1 GENERAL AIMS

- To understand the inter-relationship between ApoE and the cholinergic enzymes (AChE, BuChE and ChAT) along with APOE4 and BCHE-K genotype in dementia patients, especially in AD and DLB.
- To study the association of ApoE and cholinergic enzymes with cognitive decline and other AD related dementia markers such as Aβ, Tau and APP in AD and DLB patients.
- To study the underlying mechanism behind the interaction of ApoE, Aβ and BuChE in dementia.

2.2 SPECIFIC AIMS

2.2.1 Study 1

- To investigate the relationship between the levels of ApoE protein and neurodegenerative markers in CSF of patients with DLB, PDD, PD without dementia (PDND) and healthy controls (HC).

2.2.2 Study 2

- To explore, whether ChAT exist in extracellular fluids, it sources, if it is functionally intact, if it is capable of maintaining a certain extrasynaptic ACh level and to study the interrelationship of soluble ChAT with markers of inflammation and immune system.

2.2.3 Study 3

- To study the distribution of BCHE-K genotype and its association with APOE4 genotype and cognitive decline in AD and DLB patients.

2.2.4 Study 4

- To study ApoE and cholinergic markers in CSF of AD and DLB patients, along with their genotypes (APOE4 and BCHE-K) and their association with other CSF markers representing AD pathology such as sAPP, Aβ and Tau.

3 MATERIALS AND METHODS

3.1 PATIENT COHORTS

3.1.1 Study 1

The patient cohorts used in this study consisted of five healthy controls (HC) and 28 patients with Lewy body disorders (nine with DLB, nine with PDD and 10 with PDND) who fulfilled the clinical criteria for PD and DLB [37, 87]. The patients were recruited by experienced neurologists from the Neurodegenerative Department, University of Tuebingen (Tuebingen, Germany), using a standardized clinical examination procedure, which was expected to have an accuracy of more than 90% for the correct diagnosis [88]. In addition, all the patients underwent an extensive neuropsychological test battery. The diagnosis of PDD was based on the clinical diagnostic criteria, DSM-IV [89] indicating cognitive impairment in at least two domains that included memory dysfunction and the clinically rated impact on activities of daily living functioning. The clinical ratings were based on the reports from either the patients or caregivers regarding the daily living dysfunction caused primarily due to cognitive worsening. The differentiation of DLB from PDD was done with the 1-year rule. In other words, PDD was diagnosed, if dementia occurred later than 1 year after the first occurrence of motor symptoms and DLB was diagnosed if dementia occurred no later than 1 year after the first occurrence of motor symptoms or even preceded motor symptoms [37]. The participants also underwent imaging with MRI shortly before the study to exclude atrophy and severe deep white matter lesions, which are typical signs for vascular pathology. All participants underwent the assessments reported here within the time frame of 1 month [90]. The demographic details of the patients included in this study are reported in **Table 2.**

3.1.2 Study 2

We prepared nine pooled samples of plasma and CSF from patients with defined APOE and BCHE genotypes by pooling equal volumes of at least three samples with identical genotypes, from 179 patients who had a clinical diagnosis of mild to moderate AD recruited form Geriatric Clinics in Karolinska University Hospital, Huddinge, Sweden or from Pitea River Valley Hospital, Sweden. The selection and diagnosis of patients was made using an exclusionary criterion- absence of diagnosis of any other dementia, in accordance with the NINCDS-ADRDA [36]. All the samples were those collected prior to treatment with ChEIs. More details regarding the study population is reported in [58].

Brain tissues were obtained from five AD patients (mean age 78±5 years, postmortem delay 26±2 hours, nonsmoking) and five non-AD controls (mean age 82±2years, postmortem delay 21±4 hours, nonsmoking) provided by Netherland Brain Bank.

The study also included CSF samples from 10 patients who were diagnosed with multiple sclerosis (MS) and 11 patients with other non-inflammatory neurological conditions (OND). These patients were those who were attending the Neurology clinic, Karolinska University Hospital, Solna. The clinical examinations were performed by experienced specialists in neurology and the MS patients were diagnosed based on the McDonald criteria [91]. The MS patients were also evaluated with the Expanded Disability Status Scale (EDSS) at the time of sampling by a certified rater [92].

The OND group of patients did not have any inflammatory neurological/psychiatric conditions when assessed with MRI scan and did not have any signs of inflammatory activity in CSF. No evidence of concomitant diseases such as infections was found in these patients. Corticosteroids had not been given within three months of sampling.

Table 2. Demographic details of patient cohorts in Study 1

Diagnosis group	HC	DLB	PDD	PDND	p-value
N	5	9	10	10	
Age at PIB-PET (years)	66.0 (56-80)	69.0 (62-76)	69.5 (62-80)	66 (55-83)	
Gender (F/M)	3/2	2/7	6/4	2/8	
APOE4 (+/-)	0/5	6/3	3/7	1/8	#p<0.020
Hoehn and Yahr stage	-	2 (2-3)	2 (2-3)	2 (1-4)	
Age at onset of Parkinsonism (years)	-	66 (59-73)	59.5 (55-70)	56.0 (49-75)	#p<0.030
Duration of Parkinsonism (months)	-	36 (24-60)	108 (60-168)	96 (24-204)	#p<0.006
Age at onset of Dementia (years)	-	67 (59-73)	67 (61-78)	-	
Duration of Dementia (months)	-	36 (24-48)	24 (15-36)	-	#p<0.025
MMSE	29.0 (27-30)	19 (18-27)	23.5 (19-30)	28.5 (26-30)	*p<0.0001

Values are displayed as median (range). #p value between DLB, and patients with PDD and PDND.
*p value between groups with dementia (PDD and DLB) and groups without (PDND and HC).

3.1.3 Study 3

The overall study cohort consisted of 368 ethnic Norwegians who were recruited as a part of two large ongoing studies in Norway namely DemVest and TrønderBrain study. Of these individuals, there were 108 were AD patients, 174 were DLB patients and 86 were Controls. All AD and a portion the of DLB patients belonged to the DemVest study, and had been recruited from the dementia clinics in western Norway [93]. The additional DLB patients and controls belonged to TrønderBrain study and were recruited through hospitals, outpatient clinics, nursing homes, or from local care authorities in central and southwestern parts of Norway. The control subjects included caregivers not genetically related to the patients, and other elderly volunteers recruited from societies for retired people in the same area, all without first-degree relatives with dementia. All the controls, were healthy for their age, and when tested, did not have cognitive deficits [67, 94]. The diagnosing of AD and DLB patients was performed as described in [93, 94]. The gross cognitive screening in these patients was performed with MMSE and the DemVest patients had an annual cognitive assessment with MMSE for up to five years. **Table 3** provides information regarding the baseline clinical and demographic characteristics of the study cohorts.

Table 3. Baseline clinical and demographic characteristics of subjects in Study 3

DIAGNOSIS	**DLB**			**AD**	**CONTROLS**
CENTRES	**DEMVEST (N=75)**	**TRØNDERBRAIN (N=99)**	**TOTAL (N=174)**	**DEMVEST (N=108)**	**TRØNDERBRAIN (N=86)**
FEMALES (%)	47	43	44$^{\&\&\&}$	71	49 **
AGE (YEARS)	75.4±7.4	76.9±7.8	76.3±7.6	74.6±7.8	73.9±5.9$^{\#}$
DISEASE DURATION (YEARS)	3.3±2.3$^{\$\$}$	4.6±3.1	4.0±2.9$^{\&\&\&}$	2.5±1.8	NA
MMSE	23.6±3.3$^{\$\$\$}$	19.3±7.0	21.3±6.0$^{\&\&\&}$	24.0±2.3	NA

$^{\&\&\&}p<0.001$ are significant differences between AD and DLB; $^{**}p<0.01$ are significant differences between controls and AD; $^{\#}p<0.05$ is significant difference between Controls and DLB; $^{\$\$\$}p<0.001$; $^{\$\$}p<0.01$is significant difference between Demvest and Tronderbrain DLB. Values are given as the mean ± standard deviation.

3.1.4 Study 4

This study includes a subgroup of about 33 AD patients, 10 DLB patients and 13 healthy controls from the DemVest study, which was described in **Study 3**. The diagnostic procedures of these patients were similar to those described in **Study 3**. The healthy controls were recruited from the patients visiting the clinic for elective orthopedic surgery or

neurological outpatient assessment, who did not have any known neurological diseases and were confirmed cognitively normal after an interview and cognitive screening with MMSE [95, 96]. **Table 4** gives detailed information regarding the study cohorts in **Study 4**.

Table 4. Characteristics of the study cohorts in Study 4

DIAGNOSIS	AD (n=33)	DLB (n=10)	CONTROL (n=13)
Female (%)	70	40	67
Age	74(50-89)	73(55-84)	78(66-84)
BL-MMSE	24(20-29)***	26(22-28)$$	28(26-30)
BCHE-K Carriers (%)	48	30	NA
APOE4 Carriers (%)	73	70	NA

The values are given as median (range) or number (%). ***p<0.001 and **p<0.01 represents the significant difference between controls and demented groups (AD and DLB).

3.2 ETHICAL CONSIDERATIONS

Written informed consent was obtained from the patients or their proxies, and from the control individuals involved in all four studies. The studies were conducted according to the Declaration of Helsinki and the subsequent revisions.

- **Study 1** was approved by the ethical committee from the University clinic at Tübingen, Germany (Ethik-Kommission an der Medizinischen Fakultät der Eberhard-Karls-Universität und am Universitätsklinikum Tübingen).
- **Study 2** was approved by either the regional ethical review board in Stockholm or the research ethics committee of the south location, Huddinge University Hospital, Sweden.
- **Study 3 and 4** was approved by the regional ethical committee for health and medical research ethics in western Norway (for the DemVest Study) and by the regional ethical committee for health and medical research ethics in mid Norway (for the Trønderbrain study).

The obtained ethical permissions also included the permit to transport the samples abroad.

3.3 SAMPLE PREPARATIONS

All the samples used in the studies were collected/prepared, transported, aliquoted, diluted and stored at -80°C prior to the experimental procedure.

3.3.1 CSF collection and storage

CSF was taken from all the subjects by lumbar puncture using polypropylene tubes. Routine CSF diagnostics (cell count, albumin, immunoglobulin G level, immunoglobulin G index, and cytology) were performed. Further details regarding the CSF collection in **Study 1** is reported in [97], **Study 2** in [58] and **Study 4** in [96]. The CSF samples were centrifuged within 1 hour after collection and stored at -70°C until analysis.

The CSF from the study cohorts collected from the respective hospitals were transported to the laboratory at Karolinska Institutet, Stockholm, Sweden as per the standard transportation procedures in dry ice. The samples were aliquoted, diluted as per the desired dilutions for specific experiments and stored at -80°C. Later the diluted samples were taken out and thawed while performing the experiments.

3.3.2 Preparation of brain homogenates

In **study 2**, the cortical brain homogenates (BH) from parietal cortex of five AD patients and five non-AD controls were prepared as described previously in [56]. The pooled BH samples were prepared from each group and stored at -80°C for the experiments.

3.3.3 DNA extraction

For **study 3**, the blood samples were collected and handled according to standardized procedures, aliquoted, frozen, transported and stored at –80°C at Karolinska Institutet, Stockholm, Sweden. DNA was extracted from approximately 3mL of whole blood using the Gentra Puregene blood kit (cat no. 158389) from Qiagen according to the manufacturer's instructions and was stored at -80°C.

3.4 PROTEIN AND ACTIVITY MEASUREMENTS

Different kinds of Enzyme linked immunosorbent assay (ELISA) such as sandwich ELISA, direct ELISA and multiplex-ELISA was performed to quantitatively measure the concentrations of different proteins. The activity of enzymes was measured using a functional assay integrated with a sandwich ELISA or ELISA-like assay setup. All the ELISAs were performed either in a 96-well or 384-well microtiter plates (Nunc Maxisorb, Cat no 236366, Denmark) and the reaction was monitored at specified wavelength using an Infinite H M1000 Tecan microplate reader.

The ELISA methods used for the measurement of AChE, BuChE, ChAT and ApoE were all designed, developed and validated in our lab by the members of Darreh-Shori's group.

(i) ApoE protein levels

For ApoE measurements, all the CSF samples were diluted 50X in advance with TBS- $T^{0.2\%}$ (10mM Tris–HCl, pH 7.4; 0.2% Tween-20; all from Sigma), and used in a sandwich ELISA protocol reported previously [69]

(ii) AChE and BuChE protein levels and activity

For AChE and BuChE measurements, the CSF samples were diluted 5X with dilution buffer [TBS (10mMTris–HCl); 0.1% BSA (Bovine serum albumin); 1mM EDTA (Ethylenediaminetetraacetic acid) and 0.05%Triton X-100; All reagents were from Sigma Aldrich]. The protein level of AChE-R and AChE-S variant was measured separately using specific antibodies by ELISA-like assays [98]. The total and the functional BuChE protein concentrations were measured using a sandwich ELISA and an ELISA-like assay, respectively, as described in [58]. The AChE and BuChE activities were measured using a modification of Ellman's calorimetric method, using acetylthiocholine iodide (ATC) and butyrylthiocholine iodide (BTC) as substrates, respectively. We used ethopropazine, a BuChE inhibitor while measuring the AChE activity and BW284C51, a selective AChE inhibitor while measuring the BuChE activity [58, 98].

(iii) ChAT protein levels and activity

In **Study 2**, we developed a new unique, simple, non-radioactive and sensitive technique for determination of ChAT activity and protein concentration. Both ChAT protein and activity measurements were done in a single assay in a 384 well microtiter plate (Nunc Maxisorb, Denmark). ChAT protein and activity in CSF, BH, plasma and cell medium were measured using the new method.

The ChAT activity measurement is based on the ChAT chemiluminescence assay by [99] and we have done several modifications to improve the handling of samples, the control blanks and the detection system. We used native and denatured samples in our assay, where the denatured samples served as a control for both ChAT activity and endogenous concentration of choline in the samples.

The ChAT protein concentration was measured using sandwich ELISA. We tested the sensitivity and specificity of our ChAT sandwich ELISA using different pairs of antibodies and found that the combination of the anti-ChAT mouse monoclonal antibody (MAB 3447, R&D System, UK) as the capturing antibody and anti-ChAT rabbit polyclonal antibody (PAB 14536, Abnova Corp. Taiwan) had less unspecific binding with less variations and noise to

signal ratio compared to other antibodies. We used a recombinant human ChAT protein (R&D systems) as standards for this assay.

Since the main objective of the **Study 2** is to show the presence of extracellular ChAT, we used reagents recognizing both the N and R type splice variants and the common and peripheral ChAT isoforms, instead of reagents specific to a particular isoforms of ChAT.

A comprehensive description of this assay is described in the published study paper.

(iv) sAPPα and sAPPβ levels

The sAPPα and sAPPβ measurements in **Study 1** (with 50x diluted CSF) **and Study 4** (4X diluted CSF) were performed using the duplex ELISA kits developed by Meso Scale Discovery, Rockville, MD, USA.

(v) T-Tau and P-Tau levels

The T-Tau and P-Tau measurements in **Study 1** was performed using undiluted CSF in a duplex ELISA kit for total tau/phosphorylated tau 231 from Meso Scale Discovery, Rockville, MD, USA (96-well MULTI-SPOTAD assay: phosphorylated (Thr 231)/total tau with purified neuronal tau calibrators). The T-Tau and P-Tau 181 measurements in **Study 4** was performed using commercial sandwich ELISAs: INNOTEST hTAU-Ag and INNOTEST PHOSPHO-TAU (181P) (Innogenetics, Ghent, Belgium) respectively.

(vi) Aβ38, Aβ40, and Aβ42 levels

In **Study 1 and 4**, the Aβ38, Aβ40, and Aβ42 levels in the CSF samples were determined using a triplex ELISA kit from Meso Scale Discovery.

3.5 SNP GENOTYPING

The APOE4 genotyping for **Study 3 and 4** was performed as described in [67]. The BCHE-K (rs1803274) genotyping in **Study 3 and 4** was performed with purified DNA using the TaqMan SNP genotyping assay kit (cat no. 4351379, Applied Biosystems), and was confirmed in whole blood using the TaqMan Sample-to-SNP assay kit (cat no. 4403087, Applied Biosystems), according to the manufacturer's instructions, using a StepOne Plus thermal cycler (Applied Biosystems) as described previously [58].

3.6 DOT BLOT AND WESTERN BLOT ANALYSES

The presence of ChAT in CSF, plasma and BH was first tested by a simple dot-blot technique using anti-ChAT antibodies MAB 3447 and PAB 14536 in **Study 2**. This was then confirmed using Western-blot, which is more specific than dot-blot analysis, and also helped in identifying the different possible molecular forms of ChAT.

3.7 SUCROSE-DENSITY GRADIENT ULTRACENTRIFUGATION AND SEDIMENTATION ANALYSIS

Further evidence and insights about different molecular forms of ChAT were assessed by sedimentation analysis. Briefly, protein content of nine different pooled CSF samples were separated by sucrose- gradient sedimentation technique and ultracentrifugation at 1650006g in a continuous sucrose gradient (5–20% w/v) for 18 hours at 4°C in a Beckman rotor (SW 41 Ti) as described previously [56]. The sucrose gradients (10 mL/tube) were prepared in TBS (Tris-HCl, 10 mM, pH 7.4, containing 150 mM NaCl and 50 mM MgCl2) and the pooled CSF (0.5 mL) or BH (0.3 mL) samples were applied on the top of the gradients. Approximately 50 fractions were collected from the bottom of each tube. Concentration of ChAT protein in the collected fractions was then determined by our sandwich ELISA. Enzymes of known sedimentation coefficient, bovine liver catalase (11.4S ≈ 250 kDa) and calf intestinal alkaline phosphatase (6.1S ≈ 140 kDa), were used in the gradients to estimate the sedimentation coefficient and Mw of different molecular forms of ChAT, using the following equations.

Sedimentation coefficient of protein of interest, (ChAT in this case)(in Svedberg units, S), $\mathbf{S_p = [(N_t-N_p)/(N_t-N_r)] * S_r,}$

Where,

$\mathbf{N_t}$ = Number of total fractions collected from the gradient.

$\mathbf{N_p}$ = Number of the fraction containing the protein of interest

$\mathbf{N_r}$ = Number of the fraction containing the reference protein (Catalase or alkaline phosphatase)

$\mathbf{S_r}$ = Sedimentation coefficient of the reference protein (Sedimentation coefficient of catalase is 11.4S and alkaline phosphatase is 6.1S).

The molecular weight of protein of interest, $\mathbf{Mw_p = (Mw_r * S_p^2)/ S_r^2}$

Where,

$\mathbf{Mw_r}$ = Molecular weight of the reference protein.

3.8 IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

In **Study 2**, the spleen cells (10^6 cells/mL) from C57BL/6 mice were stimulated with lipopolysaccharides (LPS, 25 mg/mL) or with anti-CD3 (clone 2C11 at 5 mg/mL). The cell cultures were run in parallel in duplicates in RPMI1640 (HyClone Cat no SH3009601), supplemented with 10% fetal calf serum, 2 mM L- glutamine, 50 U/ml penicillin, 50 g/ml streptomycin, 10 mM HEPES, 1 mM sodium pyruvate and 50 mM 2-mercaptoethanol.

The cells and medium were collected after 24 and 48 hours for analysis. The cells were washed 3 times with PBS, and mounted on adhesion slides (ER-202W-AD-CE24, Thermo Scientific). The cells were fixed and prepared for the subsequent confocal microscopy as described in [100] with minor modification. The cells were permeabilized with 0.2% saponin, instead of Triton-X100, for 15 min each. A rabbit polyclonal anti-ChAT antibody (AB143, Millipore, 1:100) was used for the immunocytochemistry of ChAT in the mice lymphocytes.

3.9 HUMAN ASTROCYTE CULTURES

For **Study 2**, primary human astrocytes were obtained from ScienCell Research Laboratories (Cat no 1800) and were cultured in astrocyte medium (ScienCell Cat no 1801) in poly-L-lysine-coated T-175 flasks, and handled according to manufacturer's protocols. Confluent cells were then detached, and seeded in a 48-well plate ($3x10^5$ cells/well), and left in medium overnight to recover. Following this, the cells were stimulated with ACh or tumor necrosis factor-α (TNF-α) alone, ACh+TNF-α or left unstimulated as control. ACh concentration varied between 1 mM to 10 mM and TNF-a concentration was kept constant at 20 ng/ml. The astrocytes were stimulated for 24 hours, after which the cells were lysed for RNA extraction and subsequent reverse transcriptase-PCR (RT-PCR) expressional analysis.

3.10 RNA PREPARATION AND RT-PCR

In **Study 2**, messenger ribonucleic acid (mRNA) was extracted from the astrocytes and then complementary deoxyribonucleic acid (cDNA) was synthesized using RT (reverse transcriptase)-PCR kits from Qiagen and Bio-Rad according to the manufacturer's protocols. Quantitative gene expression was done by real-time PCR using a three-step PCR protocol and Bio-Rad CFX manager software (version 2.1, Bio-Rad, Hercules CA). The primers and probes for the experiment was designed with primer blast (www.ncbi.nlm.nih.gov/tools/primer-blast), and checked for specificity with melt-curve analysis. The housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was

used to normalize the mRNA expression levels of the studied transcripts. Normalized expression levels were calculated with Bio- Rad CFX manager.

3.11 NEUROIMAGING AND COGNITIVE MEASURES

The regional cerebral glucose metabolism and the Aβ load in the brain were measured using ^{18}F-FDG-PET and ^{11}C-PIB PET respectively in **Study 1,** which was done in the facilities at Nuclear Medicine and PET Center, University of Tuebingen, Tuebingen, Germany. DatScan using SPECT was available for the DLB subjects in **Study 3 and 4, w**hich were performed at the respective centers belonging to DemVest and Trønderbrain study.

Cognitive assessment was performed using MMSE, Boston Naming Test and other neuropsychological tests in all the studies.

3.12 STATISTICAL ANALYSIS

The statistical analyses were performed using Statview (SAS Institute Inc.) for all the studies. Additionally in the **Study 3**, the R-program for statistical computing was used.

In **Study 1, 2 and 4,** the significance of results between the groups was analyzed using a analysis of variance (ANOVA, with a significance level chosen as of $p< 0.05$, and was followed with Fisher's protected least significant difference post hoc analysis. Due to the small group size as well as the sensitivity of parametric tests to outliers, we double-checked the significant differences between the groups by both visual inspection and non-parametric Mann-Whitney tests. A two-tailed correlation z test (Statview) and nonparametric Spearman's rank correlations were used for the correlation analyses, which were then visualized graphically using simple regression plot.

In **Study 3,** Chi-square and Fisher exact tests were used to compare the genotype frequency and gender differences between the groups. The age, disease duration, and MMSE variable were compared between the groups using one-way ANOVA. Longitudinal analyses were performed with a linear mixed effects model, adjusting for covariates (age and gender) using R-program for statistical computing. A model with random intercept and slope, and Independently Identically Distributed (IID) residuals was used to produce a fit to the data. Further explorative analyses were performed using repeated-measures ANOVA.

3.13 METHODOLOGICAL APPROACH

The methodological approach for each study is represented as a flowchart in **Figure 5 (Study 1), Figure 6 (Study 2), Figure 7 (Study 3) and Figure 8 (Study 4)**

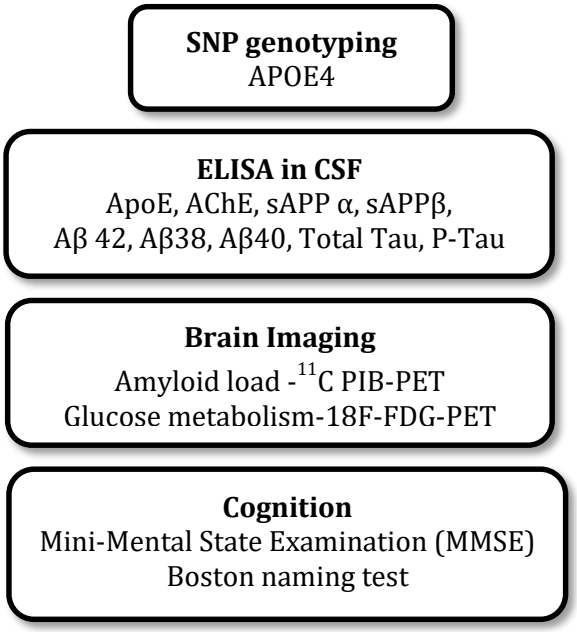

Figure 5. Methodological approach in Study 1

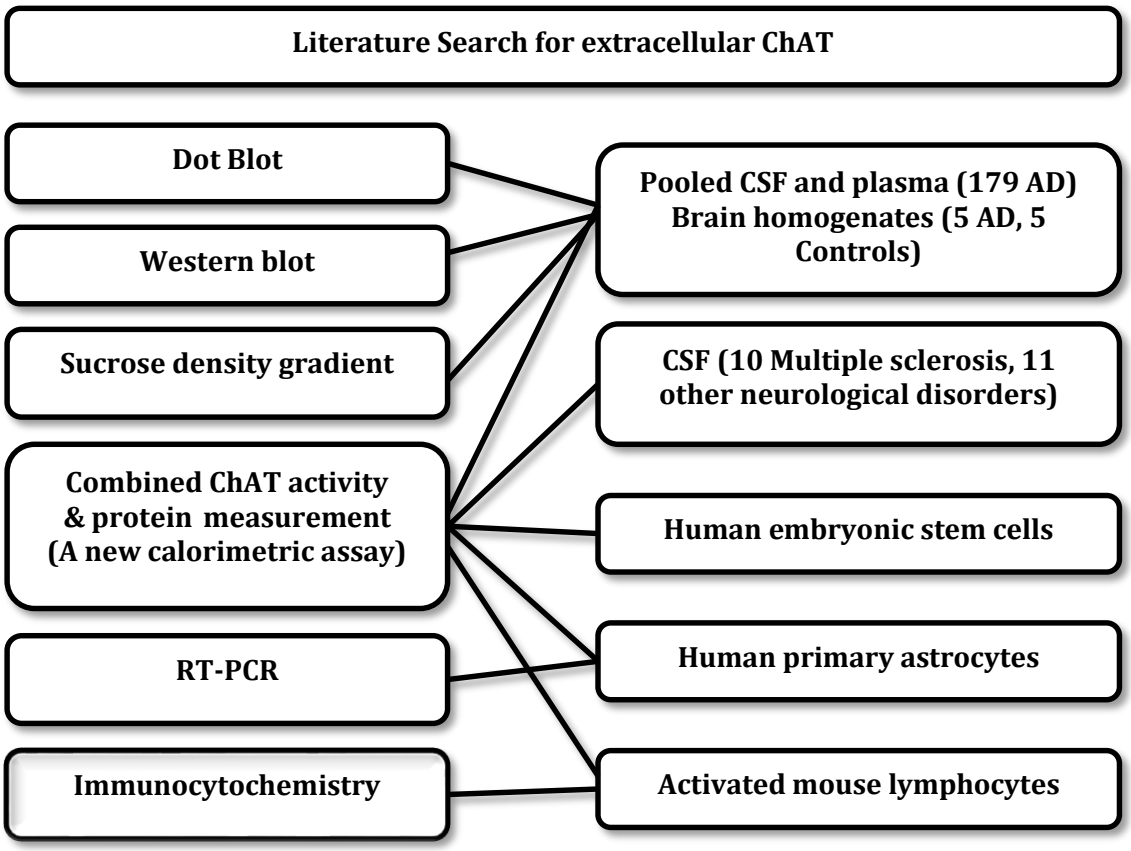

Figure 6. Methodological approach in Study 2.

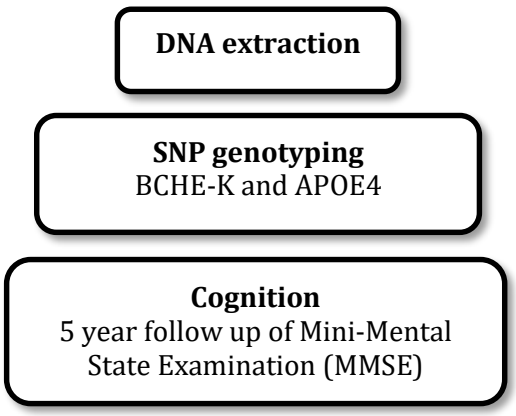

Figure 7. Methodological approach in Study

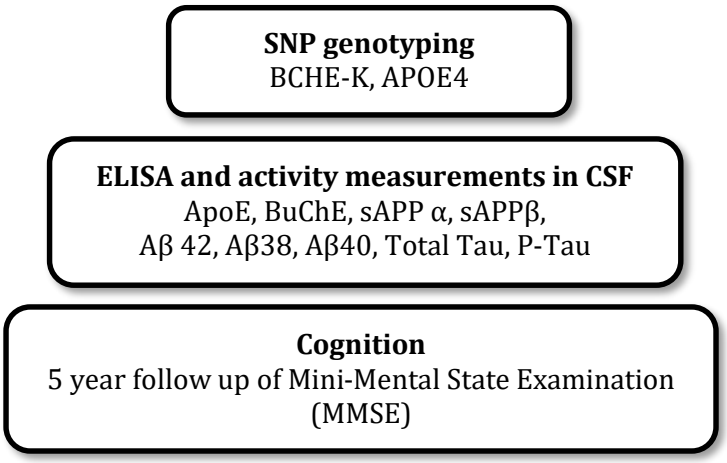

Figure 8. Methodological approach in Study 4.

4 RESULTS AND DISCUSSION

This section of the thesis highlights the most important findings from **Studies 1, 2, 3 and 4**. Please refer to the study papers for details on all findings.

4.1 EVIDENCE OF EXTRACELLULAR CHAT

About a century of research in the cholinergic field has implied that ChAT is solely a cytosolic enzyme of the cholinergic neurons. However, from our **Study 2**, we found that, apart from being an intracellular enzyme, ChAT also exists extracellularly in CSF and plasma. We also showed that it is not only expressed but actively secreted by various cells, such as human embryonic stem cells, human primary astrocytes and mouse lymphocytes. This study, for the first time evinces astrocytes as cholinergic cells.

The dot-blot analysis with different specific antibodies for human ChAT, provided compelling evidence for the presence of ChAT in CSF, plasma and brain homogenate (BH) **(Figure 9a, b)**. We further performed western blot analyses and confirmed the presence of soluble ChAT. These analyses also revealed for the first time that ChAT may exist as various molecular forms, most likely composed by assembly of its monomeric globular subunit (G1) in a similar fashion to its counterpart enzymes, ChEs in CSF, plasma and brain homogenate **(Figure 9c)**. Using the sedimentation analysis technique, we were able to confirm this and further characterize the different molecular forms of ChAT in CSF as G1, G2, and G4 with the corresponding molecular weights of 50–70 kDa, 100–140 kDa and 200–300 kDa, respectively **(Figure 9h)**.

We then developed a new sensitive, non-radioactive, simple and integrated ChAT assay, which sequentially measures first the activity and then the protein level of the enzyme **(Figure 10)**. The assay results showed that plasma had about 50 fold higher ChAT than CSF and BH **(Figure 10a, b)**.

The stimulation experiment on mouse lymphocytes and human primary astrocytes showed that levels of ChAT protein secreted into the culture medium increases when activated **(Figure 10c)**. This suggests that soluble ChAT might be required for *in situ* synthesis of ACh in extracellular fluid, and thereby for autocrine and/or paracrine ACh signaling.

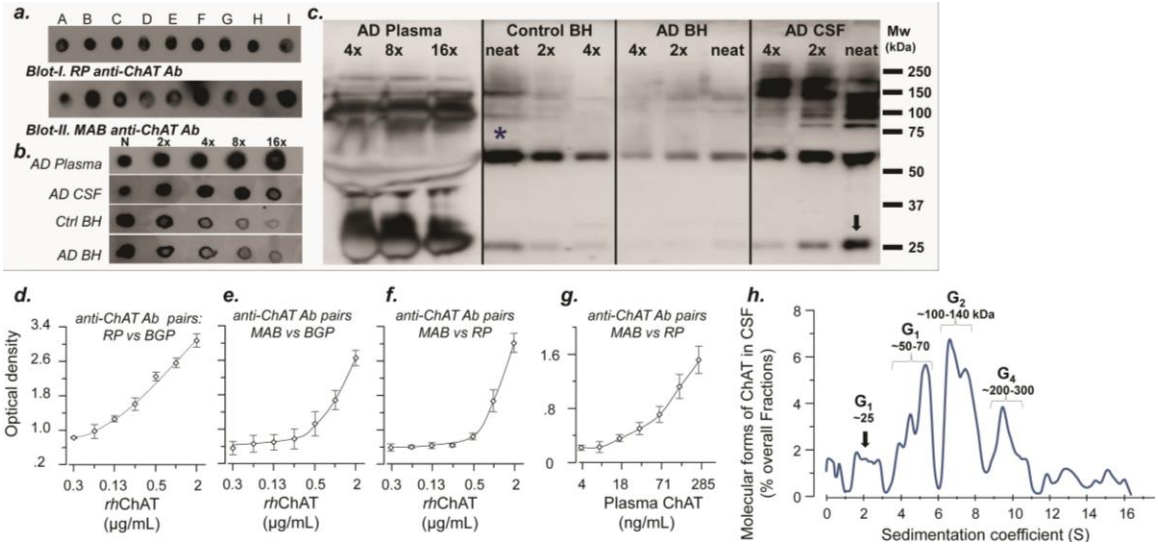

Figure 9. Detection and characterization of extracellular ChAT protein in human plasma and CSF. (a) Dot-blot analysis of nine different pooled CSF samples using two different antibodies. (b) The relative amount of ChAT in plasma or CSF, compared to brain homogenates (BH) of AD or control. At 16-fold dilutions, the immunosignals are much stronger in plasma and CSF compared to BH. This demonstrates the relative abundance of the ChAT protein in extracellular fluids. (c) Western-blot characterization of molecular form of ChAT in plasma, CSF and BH. The major detected protein band (*) in the BHs corresponds to a ChAT protein with a Mw of 65 kDa. In addition, there are several detected heavier molecular forms of ChAT in the CSF. All samples were loaded on one gel. Note also that the amount of the,65 kDa ChAT was so high in plasma that it distorted the gel downward. (d–g) Combinatorial sandwich ELISA results for identification of extracellular ChAT using three different antibody pairs for recombinant human ChAT (rhChAT), and a pooled plasma sample (g) calibrated for ChAT protein concentration using the rhChAT and ELISA setup in (f). (h) Further characterization of the molecular forms of CSF ChAT in consecutive fractions of pooled AD CSF samples separated by sucrose-density gradient technique, and the subsequent quantification of ChAT by sandwich ELISA. The graph represents the average of nine different pooled CSF. This independent analysis provides an identical pattern of molecular forms detected by Western analysis. Due to lack of prior reports, we used analogous terminology, which is used for the counteracting cholinergic enzymes, AChE and BuChE, that is, Gn, where n denotes the number of globular subunits in each detected molecular form of ChAT in CSF. The molecular weights are calculated based on the known Mw of two internal standard proteins. In all dot-blot analyses, 2 mL of each sample (neat or diluted) was used. In the Western blot analysis, each lane was loaded with 15 mL of a mixture, containing 10 mL of sample (neat or diluted) and 5 mL of a 6x concentrated reducing Laemmli loading buffer. All ChAT protein quantifications were done with the ELISA antibody pair's combination in (g). Anti-ChAT antibodies: RP=rabbit polyclonal antibody (Ab), BGP=biotin-labeled goat polyclonal Ab, MAB=mouse monoclonal Ab.

4.2 MAINTENANCE OF ACH EQUILIBRIUM BY EXTRACELLULAR CHAT

Acetylcholine is an anti-inflammatory signaling substance that may regulate activity of various immune cells. However, this hypothesis poses a dilemma since ACh is a very unstable molecule and thereby very unlikely to be effective over long-distances. Our finding of extracellular ChAT in **Study 2** provides a plausible explanation and evidence for this

missing link of such long-distant action of ACh in the cholinergic anti-inflammatory pathway of central and peripheral nervous system.

The immunocytochemistry results from the stimulation experiment on the activated mouse lymphocytes added further evidence that ChAT rather than ACh is secreted by these cells. We hypothesized that the function of soluble ChAT is to maintain the equilibrium level of extracellular ACh, by continually resynthesizing ACh *in situ*, allowing ACh to have a tuned immuno-modulatory function, e.g., on functional status of lymphocytes and other cholinoceptive cells such as astrocytes.

This hypothesis also allows us to explain another observation reported by us. Namely, in our recent study [69] we had shown that in AD CSF, Aβ peptides along with ApoE interacts

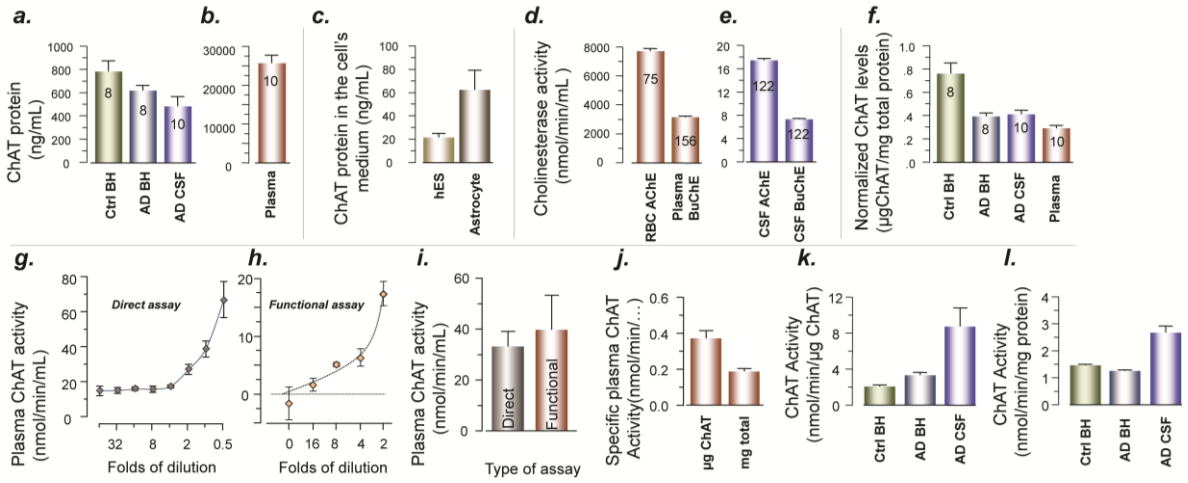

Figure 10. The extracellular ChAT protein is functionally intact and physiologically balanced in both plasma and CSF. (a–b) The quantity of the ChAT protein determined by ELISA in pooled brain homogenates of control brains (Ctrl BH) and AD brains (AD BH), AD CSF and plasma. The concentration of ChAT in the plasma is 50-fold higher (b) than the CSF samples (a). (c) The amount of ChAT in the cell-culture medium of human embryonic stem cells (hES) and human brain primary astrocytes, indicating that these cells readily released ChAT into the medium. (d–e) illustrate , 300-fold higher overall activity of the counteracting enzymes, red blood-cell acetylcholinesterase (RBC-AChE), and plasma BuChE (d) compared to the AChE and BuChE activities in the CSF (e). These observations may suggest that ChAT levels in plasma and CSF (a–b) is balanced to the corresponding physiological levels of AChE and BuChE activities. (f) The amount of ChAT normalized to the total protein in the samples. (g–h) Two new, alternative, colorimetric assays of ChAT activity confirmed that the plasma and CSF ChAT are functionally intact. ChAT activity was measured directly (g) or after immunocapturing of the ChAT protein (h) into wells of microtiter plates coated with mouse monoclonal anti-ChAT antibody in a functional ELISA setup. (i) Comparison of measured ChAT activity by these two approaches, indicating that both approaches provide essentially similar levels of ChAT activity in pooled plasma samples. The functional assay also excludes any possible contribution or interference of other plasma constituents in the synthesis of acetylcholine (e.g., by carnitine acetyltransferase), since ChAT protein is first immunoadsorbed and the other plasma constituents are washed away before the substrates and other necessary reagents are added. (j–l) The plasma, CSF and brain ChAT activities normalized to quantify ChAT protein or the total protein in the samples. The digits in the column bar represent the number of samples that were analyzed.

with AChE and BuChE leading to the formation of high molecular weight BuChE/AChE-Aβ-ApoE (BAβA) complexes. These BAβACs contain hyperactive ChEs, which readily degrades ACh in the extracellular compartments. Our finding of the extracellular presence of ChAT helps to understand the equilibrating mechanism involved with this depletion in the level of ACh. This way, we proposed a native biological function for Aβ peptides. In other words, by Aβ release and reuptake, cells can shift the extracellular ACh equilibrium to low and high levels, respectively. Thus Aβ, through BAβACs, may function as a modulator of ACh signaling.

To provide some evidence for this hypothesis, we designed an ACh equilibrium assay and showed the maintenance of ACh equilibrium by ChAT in the presence of fully active and naturally occurring amounts of the ACh degrading enzymes, AChE and BuChE at both room temperature (where ChAT is sub-optimally active) and 38°C (at which ChAT is fully active **(Figure 11)**.

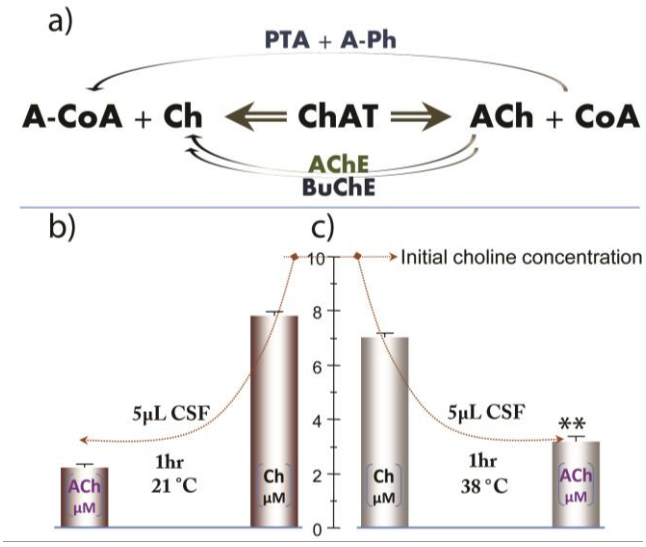

Figure 11. Extracellular CSF ChAT proteins maintain a steady- state level of ACh in the presence of naturally occurring levels of fully active cholinesterases. (a) Schematic illustration of the overall reaction setup. Pooled CSF sample was incubated for one hour with a cocktail containing 10 mM choline chloride and 50 mM acetyl- Coenzyme A (Acetyl-CoA). To avoid depletion of Acetyl-CoA, the accessory enzyme phosphotransacetylase (PTA) and acetyl-phosphate (A-Ph) were included in the cocktail to continuously regenerate the required cofactor, Acetyl-CoA. It should be noted that this setup allows an equilibrium between synthesis and degradation of ACh be reached (b) CSF ChAT maintains a steady-state ACh concentration of 2.11±0.27 mM after one hour at room temperature. However, ChAT becomes fully activated at 38uC. This is shown in panel (c), corresponding to a steady-state ACh concentration of 3.43±0.14 mM. No inhibitor of AChE or BuChE was used providing strong evidence that the ACh-synthesis by ChAT produces an ACh equilibrium level in the presence of naturally occurring amount of these ACh-hydrolyzing enzymes in CSF. **p<0.0013 compared to ACh level generated at the room temperature.

4.3 HIGH CSF APOE LEVELS IN APOE4 CARRIERS AND DEMENTIA PATIENTS

Several years of dementia research have shown a close association of APOE4 with the pathophysiological alterations in AD and other types of dementia such as DLB and PDD. In accordance with the previous studies, [16, 101, 102], the AD and DLB groups in our **Study 1 and 4** had higher number of APOE4 carriers than the non-demented groups and controls.

In agreement with our previous observations [58, 69], we found a higher level of CSF ApoE in APOE4 carriers than in non-carriers. This was confirmed in both **Study 1** and **Study 4,** which included patient cohorts from two independent study groups belonging to two different geographic regions, namely Germany and Norway, respectively (**Figure 12a, 13a)**. ApoE protein serves as a receptor ligand for the low-density lipoprotein receptor and its related receptors, which are abundantly expressed in CNS by neurons. Thus, ApoE putatively mediates the transport, redistribution, and metabolism of cholesterol and other molecules in the brain, which has a high lipid turnover [103].

A plausible explanation for higher CSF levels of ApoE protein in APOE4 carriers than non-carriers could be that a larger number of ApoE4 molecules are needed to generate similar-size lipid particles compared to ApoE3 isoprotein [104]. This suggests that astrocytes and glial cells in the brain of APOE4 carriers have to express more ApoE protein to maintain a similar supply of cholesterol to neurons as occurs in APOE4 non-carriers.

Nonetheless, the studies regarding the ApoE levels in CSF (and plasma) of APOE4 carriers and non-carriers have shown contradictory results, with some reports showing high CSF ApoE levels [58, 69, 105, 106] and others showing low levels in CSF [107, 108] and brain [109] of APOE4 AD patients .

However, it should be noted that disregarding APOE genotype, we observed a trend of higher CSF ApoE level in the dementia patients (DLB and PDD compared to the non-demented patients PDND and Controls) in **Study1 (Figure 12b).** This suggests that high ApoE protein level may be involved in evolution of dementia, in particular in the presence of APOE4 genotype, with a high protein expression rate as a causative factor, for not only AD but also for DLB and PDD.

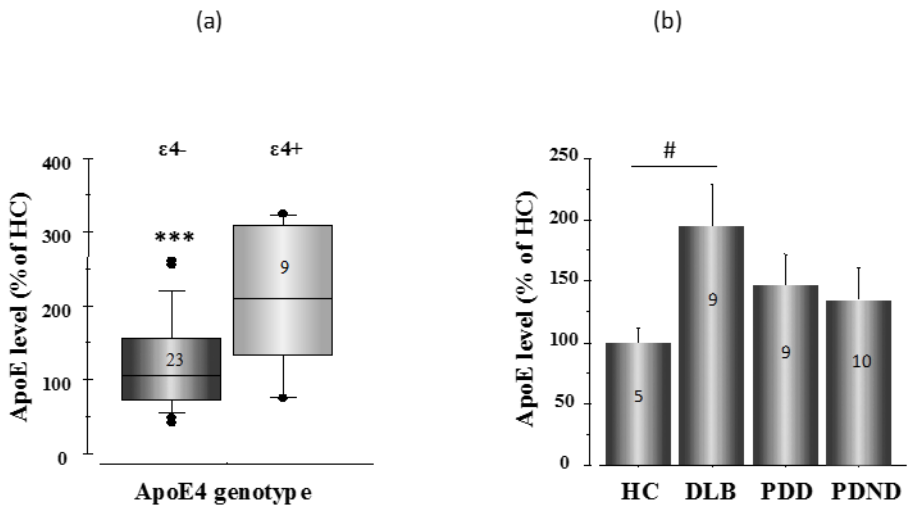

Figure 12. High cerebrospinal fluid (CSF) apolipoprotein E (apoE) protein level in patients who areAPOEε4 positive and who have dementia. (a) The apoE protein levels in the CSF of APOE ε4 carriers shows twofold higher levels than the CSF of APOE ε4 non-carriers. (b) Patients with dementia with Lewy bodies (DLB) have the highest CSF ApoE level compared with other dementia groups, regardless of their APOE genotype. ***P<0.001 indicates the difference between APOE ε4-positive and -negative groups. $^{\#}$P<0.06 indicates a trend of difference between patients with DLB and healthy control subjects (HCs) Digits indicate the number of subjects in each group. Data are presented as a percentage of HCs and standard error of the mean.

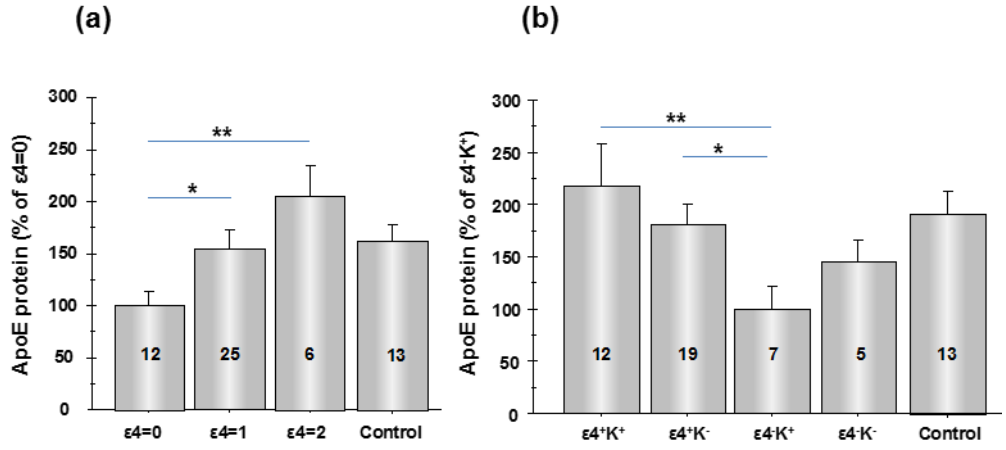

Figure 13. Apolipoprotein E (ApoE) levels in CSF of dementia (AD and DLB) patients and controls based on their APOE4 and BCHE-K genotype. (a) ApoE protein level based on number of APOE4 carriers in dementia patients and controls. ε4=0, ε4=1, ε4=2 represents the number of ε4 allele. Data are presented as percentage of ε4-noncarriers (ε4=0). **p<0.01 and *p<0.05 signify differences between ε4=0 and ε4=2, ε4=1 respectively. (b) ApoE protein level based on combined APOE4 and BCHE-K genotypes in dementia patients, and controls. $\varepsilon4^{+}K^{+}$ - patients with both APOE4 and BCHE-K genotypes, $\varepsilon4^{+}K^{-}$ - patients with APOE4 but not BCHE-K, $\varepsilon4^{-}K^{+}$ - patients with BCHE-K but not APOE4, $\varepsilon4^{-}K^{-}$ - patients without APOE4 and BCHE-K. The data are presented as percentage of $\varepsilon4^{-}K^{+}$. **p<0.01 and *p<0.05 denote the significant differences between $\varepsilon4^{-}K^{+}$ and $\varepsilon4^{+}K^{+}$, $\varepsilon4^{+}K^{-}$ respectively. Digits in the bars indicate the number of subjects in each group. Values are given as standard error of the mean. The APOE4 and BCHE-K genotypes were not available for the control group.

4.4 ROLE OF APOE4 AND BCHE-K GENOTYPES

While APOE4 genotype seems to be associated with high CSF ApoE ([69] and our findings), BCHE-K is associated with low BuChE activity in serum and CSF of AD and DLB patients [58, 110]. Consistent with these previous findings, we found that there was a significant dose dependent increase in CSF ApoE levels **(Figure 13.a)** and a trend of low BuChE activity in CSF of BCHE-K carriers than non-carriers **(Figure 14.a) (Study4)**

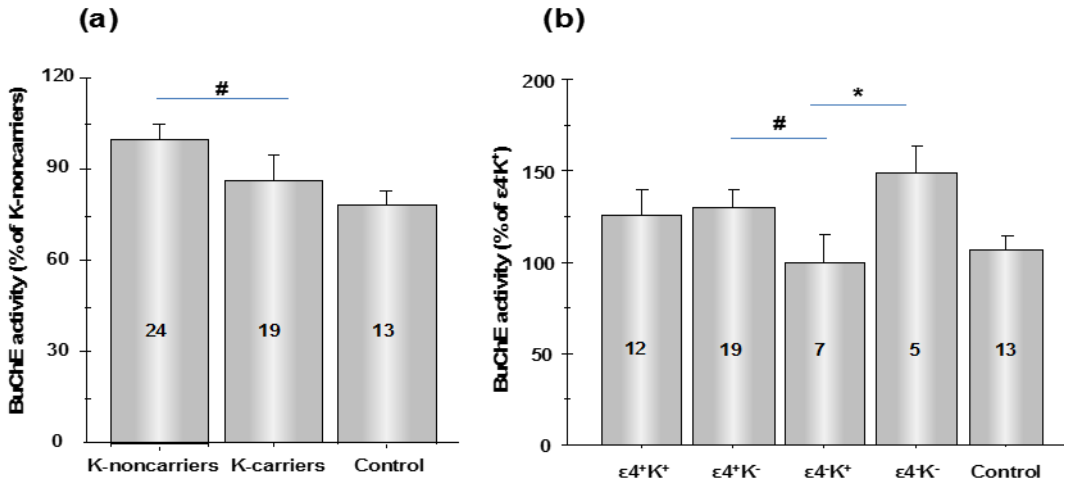

Figure 14. Butyrylcholinesterase (BuChE) activity in CSF of dementia patients (AD and DLB) and controls based on their APOE4 and BCHE-K genotype. (a) BuChE activity in BCHE-K carriers and noncarriers in dementia patients and controls. The data are represented as percentage of K-noncarriers. #p<0.09 represents a trend in difference between BCHE-K carriers and non-carriers (b) BuChE activity based on combined APOE4 and BCHE-K genotype in dementia patients and controls. $\varepsilon4^+K^+$ - patients with both APOE4 and BCHE-K genotypes, $\varepsilon4^+K^-$ - patients with APOE4 but not BCHE-K, $\varepsilon4^-K^+$ - patients with BCHE-K but not APOE4, $\varepsilon4^-K^-$ - patients without APOE4 and BCHE-K. Data are represented as percentage of $\varepsilon4^-K^+$. *p<0.05 and #p<0.1 represents the significant difference and a trend in difference between $\varepsilon4^-K^+$ and $\varepsilon4^-K^-$, $\varepsilon4^+K^-$ respectively. Digits in bars indicate the number of subjects in each group. Values are given as standard error of the mean. The APOE4 and BCHE-K genotypes were not available for the control group.

BCHE-K frequency was significantly lower in DLB patients (33.9%; p<0.01) than control subjects (51.2%), and numerically lower in AD than controls (38.9%; p=0.11) **(Study 3)**. The rate of cognitive decline was significantly higher (p=0.037) in APOE4 carriers than non-carriers, but there was no-significant difference between BCHE-K carriers and non-carriers during the five-year follow up period.

In **Study 3,** we further explored the association of APOE4 and BCHE-K genotype with cognition in AD and DLB patients. Using repeated measures ANOVA, we found that APOE4 non-carrier|BCHE-K carrier ($\varepsilon4^-K^+$) patients had a significantly slower annual decline in global cognition, assessed by MMSE test compared to those with both APOE4 and BCHE-K genotype ($\varepsilon4^+K^+$) **(Figure 15)**

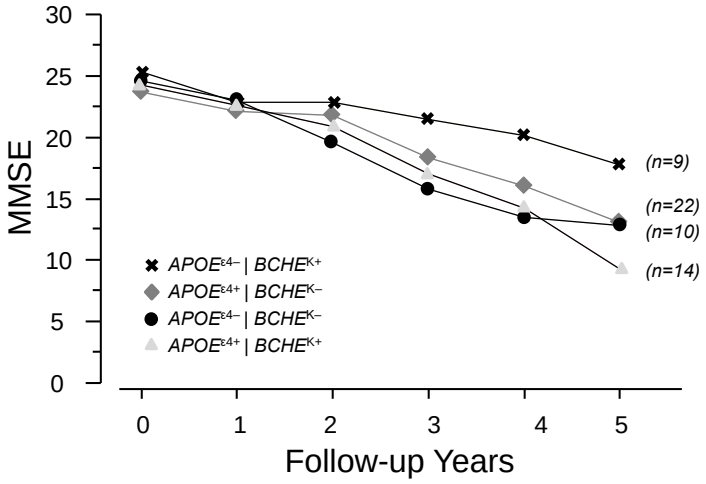

Figure 15. Cognitive decline in all AD and DLB patients based on their Apolipoprotein E ε4 (APOE4) and Butyrylcholinesterase K (BCHE-K) genotypes. All AD and DLB patients from the DemVest study are divided into four groups based on their APOE4 and BCHE-K genotypes. The lines show the mean MMSE test score in each group during the five-year follow up period. The digits in the parenthesis indicate the number of individuals in the corresponding group. Only patients with all MMSE test assessment data for the five years of follow-up were included in this analysis using repeated measures ANOVA. The patients were grouped based on the combination of APOE4 and BCHE-K genotypes. The analysis indicated a significant interaction between MMSE and the APOE4 and BCHE-K genotypes ($p<0.05$).

The outcome of APOE4 and BCHE-K genotype (alone or combined) has been investigated in several studies in AD and DLB with contradictory findings [111-115]. We did not observe any association of a slower cognitive decline with BCHE-K variant (alone, regardless of APOE genotype), although this has been previously reported in a mixed cohort of DLB and AD [116] and a large AD cohort [117]. However, in a study of patients with PD dementia, those with a combination of both APOE4 and BCHE-K had a significantly more rapid cognitive decline than the groups with either APOE4 or BCHE-K [118] indicating a potential interaction between the two genotypes. However, in our study in presence of APOE4, the BCHE-K genotype did not significantly altered the main effect of APOE4 on the annual rate of cognitive decline in the patients, although at the fifth year of follow-up the $\varepsilon4^{+}K^{+}$ exhibited divergent rate from the other groups **(Figure 15).**

Noteworthy, the APOE4 non-carrier|BCHE-K carrier patients in our study also exhibited lowest ApoE protein **(Figure 13b)** and BuChE activity levels **(Figure 14b)** in CSF compared to those who were carriers of both. This might suggest a protective effect of BCHE-K genotype through a combination effect on reduced BuChE activity (i. e. high ACh availability) and lowering ApoE protein expression. In support of this notion, a very recent study show that ApoE protein, in particular the ApoE4 isoprotein stimulates neuronal Aβ production [119] thus explaining the risk of AD by ApoE4.

In contrast, in the presence of APOE4 genotype, most likely due to high ApoE protein expression, the protective effect of the BCHE-K genotype may not be enough to compensate. It is also evident from our previous studies that APOE4 genotype through its high protein expression can modulate the phenotypic expression of BuChE protein in CSF of AD patients.

4.5 ASSOCIATION OF APOE AND BUCHE WITH OTHER AD MARKERS

The ApoE and BuChE levels in the CSF showed significant associations with the AD markers such as Aβ, Tau and sAPP.

In **Study 1**, high CSF ApoE levels were negatively associated with cognitive measures (MMSE and Boston naming tests) and cerebral glucose metabolism (^{18}F FDG-PET), CSF Aβ42 levels and AChE R/S ratio but positively with Aβ load in the brain (^{11}C PIB-PET) and CSF T-Tau, sAPPα and sAPPβ levels. In **Study 4**, once more CSF ApoE levels exhibited positive associations with Tau, sAPPα, sAPPβ and sAPPβ/α ratio in CSF (Table 6), strongly supporting the findings observed in **Study1.**

Reports indicate that APOE4-carriers exhibit accelerated age-related changes in cortical thickness and hippocampal volume, which seem to be related to the cognitive performance of patients. There are also numerous studies reporting low glucose metabolism in APOE4 carriers than non-carriers, especially among AD patients. Hence, the association of high CSF ApoE levels due to APOE4 genotype with the cognitive measures and glucose metabolism in our studies are also in line with these previous observations.

Hence, ApoE protein, and in particular the ε4 isoprotein may exert multiple roles in the pathological mechanism in dementia such as neuronal integrity, synaptogenesis and neurodegeneration as well as directly affecting neuronal Aβ production and release [119]. In addition, recent findings from our group have shown that ApoE protein prevents Aβ fibrillization in an isoform and concentration dependent manner [72].

In this context, we also found a consistent pattern of association between CSF ApoE and BuChE (overall activity, and the total and functional protein levels) **(Figure 16)**. Moreover, the BuChE activity and protein levels also showed a similar pattern of association with the CSF AD biomarkers, as was found for CSF ApoE **(Table 5).** Overall what we observed in **Studies 1** and **4** are in-line with genotypic interaction between APOE and BCHE discussed in **Study 3**. Another interesting biological link between ApoE and BuChE is that astrocytes are the source of both proteins. The astrocytes are a key regulator of the immune response in the brain, and the association between ApoE and BuChE could reflect their role in

neuroinflammation in dementia. Apart from this, both ApoE and BuChE seem to co-occur in Aβ plaques and neurofibrillary tangles further supporting the findings in the **Studies 1, 2 and 4)**. The association of ApoE and BuChE with sAPPα and sAPPβ levels in **Study 4 (Table 5)** suggests a possible role of ApoE and BuChE not only in Aβ clearance and aggregation, but also in APP processing mechanism. Hence the association of ApoE with BuChE, Aβ and sAPP is very interesting and strongly warrants further mechanistic studies.

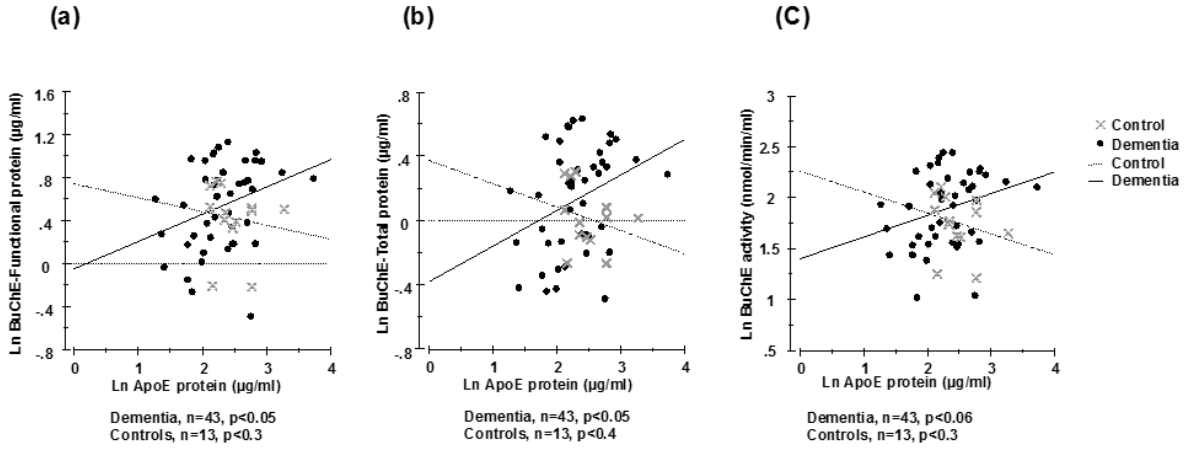

Figure 16. High Apolipoprotein E (ApoE) levels in cerebrospinal fluid of dementia patients (AD and DLB) patients is associated with high butyrylcholinesterase (BuChE) levels. In the CSF of dementia patients, the ApoE levels shows positive correlation with (a) functional BuChE protein ($p<0.05$), (b) total BuChE protein ($p<0.05$) and (c) with BuChE activity levels ($p<0.06$). In contrast in the control subjects, the pattern of association between ApoE and BuChE level was reversed, although statistically not significant [(a) BuChE functional protein ($p<0.3$); (b) BuChE Total protein ($p<0.4$) and (c) BuChE activity ($p<0.3$)].

4.6 HYPOTHETICAL MECHANISM OF PHYSIOLOGICAL AND PATHOLOGICAL ACETYLCHOLINE SIGNALING

Several previous studies by us and others, have revealed new views on possible mechanism of action and consequences of the molecular interaction between ApoE, BuChE, AChE and Aβ, the formation of BAβACs, and its role in regulation of the function of astroglial cells. This hypothesis like the well-established cholinergic anti-inflammatory hypothesis was suffering from the fact that ACh is an extremely instable signaling molecule in extracellular fluids. The investigation on the existence of extracellular ChAT was hence undertaken as a proof-of-concept in support of this hypothesis **(Figure 17).** This hypothesis is elaborated schematically by the **Figure 17**.

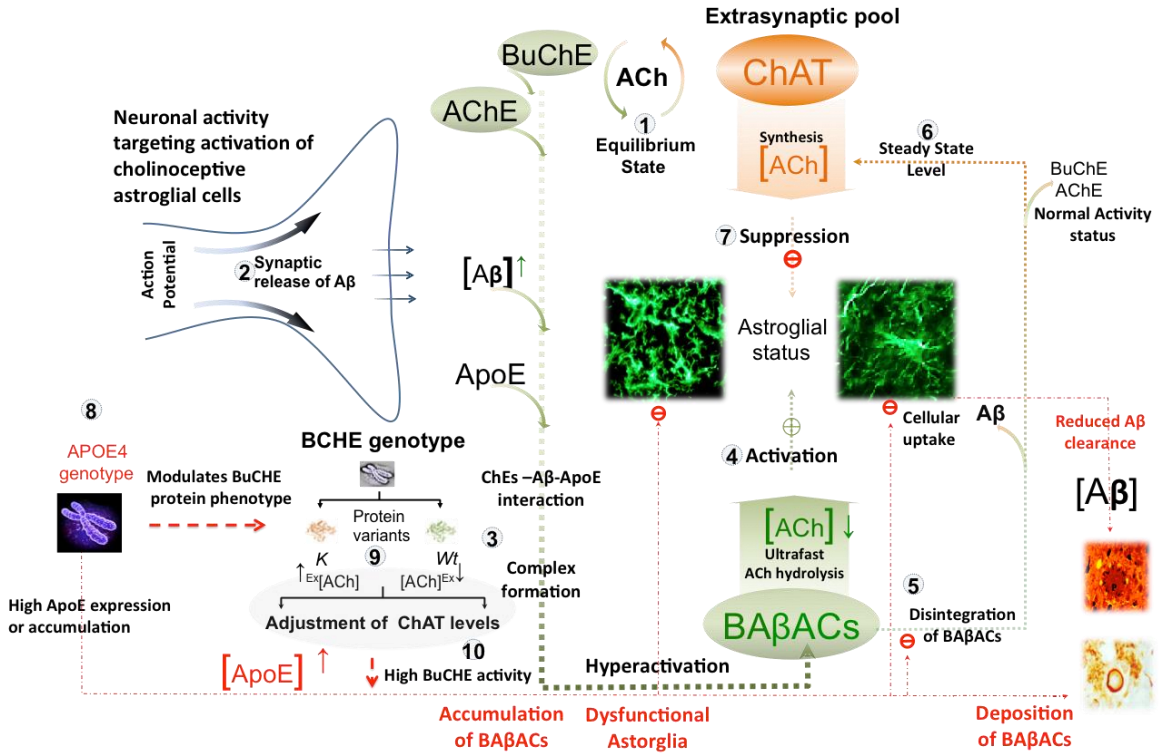

Figure 17. Hypothetical pathway for regulation of non-excitable cholinoceptive cells through distant action of extrasynaptic ACh. Extrasynaptic ACh can exert long distant regulatory effects on non-excitable cholinoceptive cells, such as microglia, astrocytes, oligodendrocytes and endothelia, mediated mainly through activation of nicotinic α7-ACh receptors. However, extracellular ACh is highly labile due to extracellular presence of the ACh-degrading enzymes, AChE and BuChE. (1) The finding here of presence of extracellular ACh-synthesizing enzyme, ChAT, proposes a model in which continuous synthesis of ACh counteracted by breakdown could maintain a steady-state equilibrium, which can regulate glial activation status. (2) This equilibrium may also be influenced by neuronal activity, since action potentials by cholinergic neurons, for example, may lead to synchronized production and release of Aβ peptides into the interstitial fluid. (3) These Aβ peptides can interact with AChE and BuChE, and temporarily form BAβA-complexes (BAβACs), in which these enzymes acquire ultrafast catalytic activity, plausibly through induction of certain conformational change in their tertiary structures. (4) The hyperfunctional BAβACs will then effectively shift the equilibrium state towards lower ACh, which in turn leads to the activation of glia with expression of, for example, complement factors. (5) Reuptake of Aβ peptides will result in disintegration of BAβACs, (6) normalization of the steady-state balance, and (7) the initial astroglial status. (8) In AD the presence of the apolipoprotein E ε4 allele (APOE4) genotype may disturb the balance additionally due to dysfunctional Aβ re-uptake (clearance) and/or high levels of ApoE protein, a common condition among APOE4 carriers. High ApoE protein will prolong the interaction between cholinesterases and Aβ, leading to gradual accumulation and deposition of BAβACs in the brain. (9) BCHE-K genotype with its low BuChE activity and BCHE-Wt with its high activity leads to high ACh and low ACh levels in extracellular compartments thereby regulating ChAT (10) APOE4 triggered high ApoE protein expression causes a phenotypic modulation of BuChE protein isoforms leading to high BuChE activity in the BAβACs. The end stage, BAβACs is deposited together with Aβ peptides in the brain as parts of the Aβ plaques and cerebral amyloid angiopathies, two hallmarks of Alzheimer's disease brain, which also explains the documented presence of BuChE, AChE and ApoE in these Aβ deposits.

In another study, which is not included in this thesis, we challenged the modulatory action of Aβ peptides on the extrasynaptic ACh signaling using neurospheres derived from human

embryonic stem cells, which we treated for 26 days with oligomeric and fibrillar Aβ preparations. One of the most striking finding in this experiment was that fibrillar, but not oligomeric Aβ, altered the release and activity of ChAT and BuChE by these cells, favoring a hypocholinergic microenvironment in the culture medium of these cells. This seemed in turn to favor the differentiation of the neurospheres' cells toward gliogenesis **(Figure 18)**. This finding hence strongly supports the notion of a close interplay between Aβ peptides and cholinergic signaling [120].

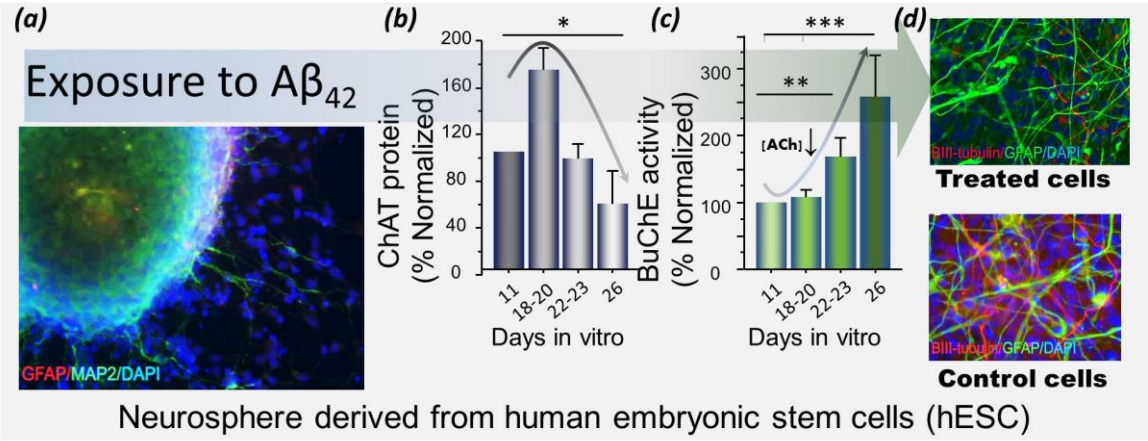

Figure 18. Treatment of neurospheres with fibrillar preparation Aβ peptides decreases ChAT protein expression and increases BuChE activity and protein expression. Neurospheres derived from human embryonic stem (hES) cells (**a**) were differentiated for 11 days in vitro (DIV) and subsequently treated once a week with different concentrations of fibrillar Aβ (fAβ). The condition cell media was collected and the protein level of ChAT (b), and BuChE activity (c) was measured at 4 different time-points (11-26 DIV) using ChAT specific sandwich ELISA and BuChE activity assay. The treated and un-treated cells were also assayed by immunocytochemistry for expression of neuronal marker (BIII-tubulin, red color), and the glial marker, glial fibrillary acidic protein (GFAP, green color). *p<0.05, **p<0.01 and ***p<0.001 indicates the significant difference between the DIV. The values are normalized to baseline at DIV11 and given as mean ±SEM. (The figure is modified from Malmsten et al, Journal of cellular and molecular medicine).

Another line of supportive evidence for the function of soluble ChAT comes from the finding that the levels of soluble ChAT in CSF were balanced by the expected reduction in the BuChE by BCHE-K genotype. In other words, we found that lower BuChE activity of the K variant protein compared to the wildtype was balanced in K-gene dosage dependent manner by a lower ChAT levels in CSF of K carriers compared to the CSF of the wild-type BCHE carrier AD patients (**Study 2)**. This might mean that the maintenance of extrasynaptic ACh level in subjects with low BuChE activity due to BCHE-K demands lower level of soluble

ChAT compared with relatively higher ACh-degrading BuChE activity due to wildtype BuChE protein variants.

This hypothetical scenario might prevail as long as ApoE protein levels and/or Aβ release and reuptake are physiologically normal, but is disrupted in pathological conditions due to for instance, abnormal ApoE protein expression by an aberrant expression of APOE gene, either duo to ε4 allele or hypocholesteremia.

Table 5. Associations of Apolipoprotein E and Butyrylcholinesterase with other Alzheimer's disease markers in Cerebrospinal fluid.

CORRELATIONS																
CSF MARKERS	**ApoE**				**BuChE Activity**				**BuChE Total protein**				**BuChE Functional protein**			
	Dementia		**Controls**		**Dementia**		**Controls**		**Dementia**		**Controls**		**Dementia**		**Controls**	
	r	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***	**r**	***p***
sAPPα	0.41	****0.001*	-0.58	*0.55*	0.17	*0.38*	0.05	*0.84*	0.19	*0.27*	-0.06	*0.92*	0.20	*0.28*	0.14	*0.55*
sAPPβ	0.20	*#0.06*	-0.55	*0.32*	0.12	*0.61*	-0.11	*0.55*	0.13	*0.41*	-0.22	*0.76*	0.12	*0.54*	-0.05	*0.84*
sAPPβ/sAPPα	-0.46	****0.001*	0.17	*0.84*	-0.05	*0.60*	-0.35	*0.69*	-0.08	*0.57*	-0.33	*0.76*	-0.11	*0.42*	-0.40	*0.23*
Ab42/Ab38	-0.32	**0.03*	0.05	*0.85*	-0.18	*0.34*	0.16	*0.95*	-0.17	*0.32*	0.33	*0.90*	-0.17	*0.30*	0.14	*0.65*
T-Tau	0.43	****0.001*	0.98	*#0.07*	0.29	*#0.08*	-0.62	*0.16*	0.29	**0.04*	-0.63	*0.26*	0.28	*#0.06*	-0.55	*0.23*
p-Tau	0.45	****0.001*	0.31	*0.55*	0.23	*#0.06*	0.42	*0.42*	0.21	**0.04*	0.53	*0.26*	0.24	**0.05*	0.34	*0.55*
Ab42/ T-Tau	-0.36	***0.01*	-0.39	*0.69*	-0.23	*0.1*	0.83	*0.84*	-0.21	*#0.08*	0.90	*0.68*	-0.20	*#0.08*	0.74	*0.55*
Ab42/p-Tau	-0.31	***0.01*	0.45	*0.23*	-0.16	*#0.08*	0.05	*0.55*	-0.11	*0.1*	0.05	*0.68*	-0.14	*#0.06*	0.11	*0.84*

****p<0.001, **p<0.01, *p<0.05 represents the significance in correlations. #p represents the trend in correlations based on Spearman rank correlations.*

5 LIMITATIONS

The major limitation in our **Studies 1 and 4** are that the patient cohorts were relatively small and hence larger sample size in each group is needed to confirm results from these studies. The small sample size might also be the reason why some statistical tests showed (only) borderline-significant results, which may fail in a more rigorous post hoc correction for multiple comparisons.

All the patients from the DemVest study **(Study 3 and 4),** Trønderbrain study **(Study 3)** and from Tuebingen hospital **(Study 1)** were those coming to the clinics for routine diagnosis and hence some of them were undergoing treatment with ChEIs, L-Dopa and other medications. Therefore, the observations from these studies should be interpreted with caution. Despite the possibility for confounding effects of such medications, the core findings could be confirmed between the studies with reasonable certainty, since the pattern of the observations were in-line with previous reports in other studies on AD and DLB patients by us and others.

The diagnoses of the patients were based on their clinical assessment. Most of the DLB subjects in **Study 3 and 4** had AD-type pathology. Hence there could be a chance of misdiagnosis especially for DLB as AD. Although, the findings in **Study 3** were adjusted for the effect of age and gender, the observations from other Studies were not adjusted for these covariates due to small sample size.

Study 2 is the only study reporting the presence of extracellular ChAT and its role in maintaining ACh equilibrium. Although we made every possible challenge that we could think that might have biased the findings, all the evidences come from our group and hence need to be confirmed by others. Some of the findings in this study are based on pooled CSF, plasma and BH from AD and control patients, which aimed at addressing the presence of soluble ChAT. Analyses on individual samples were run in a small group of patients with multiple sclerosis and their matched controls as a challenge to possible involvement of soluble ChAT in inflammation. A reported study and several ongoing studies by our group are underway to address changes in the plasma and CSF of AD, DLB, PDD, MCI and SCI, before or following short- and long-term treatment with ChEIs. These studies should provide additional insight on the function of soluble ChAT and its suitability as a biomarker in dementia and or neuroinflammatory disorders.

6 CONCLUSION

The findings in this thesis provide evidence from various angles for the presence of biological and molecular interaction between ApoE, cholinergic enzymes (ChEs, ChAT), and CSF AD biomarkers (Aβ, sAPP and Tau), as well as genotypic interaction between APOE4 and BCHE-K in dementia, especially in AD and DLB. This thesis also elucidates mechanistic information on a plausible mechanism behind the interference of ApoE with the extracellular function of BuChE and ChAT.

With the findings from our all four studies we could draw the following conclusions.

The results of two independent study cohorts **(Studies 1 and 3)** confirm that abnormally high level of ApoE in CSF is triggered by the APOE4 genotype, and may, hence, act as a driving force in the pathophysiological events of, not only AD but also the Lewy body associated dementia disorders (DLB and PDD). The results of **Study 4** confirm the notion of molecular interaction between ApoE and cholinergic enzymes at the genetic level.

The **Study 2** in this thesis provides compelling evidences for the presence of high levels of ChAT in extracellular fluids, about a century after the discovery of this enzyme by Nachmansohn and Machado [49] This study also led to development of a novel high-throughput quantitative assay for simultaneous measurement of ChAT activity and protein levels in practically any biological fluids. This assay allowed us to formulate, and challenge a new hypothesis about a possible function of soluble ChAT, viewed as a missing link for the well-established long distant hormone-like action of ACh. The study proved that CSF ChAT by continuous renewal of ACh could establish certain ACh equilibrium, despite the presence of the two fastest known ACh hydrolyzing enzymes, AChE and BuChE. This study also for the first time showed that human primary astrocytes are not merely cholinoceptive (responsive to ACh), but also that they may acquire cholinergic phenotype on demand and release soluble ChAT. Moreover, this study showed that lymphocytes release ChAT rather than ACh, and that together with astrocytes may be the source of soluble ChAT in circulation and in CNS, respectively.

7 FUTURE PERSPECTIVES

The studies included in this thesis demonstrate the importance of investigating the mechanism of interaction between ApoE, BuChE and ChAT in AD and DLB patients. Hence, findings warrant more future studies with large multi-centered patient cohorts to confirm and expand the findings. The measurements of ApoE, BuChE and ChAT in matched CSF and plasma from dementia patients and controls along with their APOE4 and BCHE-K genotype should enable deeper insights about their contribution in the pathology of dementia and especially in AD and DLB. Such studies, along with neuroimaging and clinical parameters, with multivariate analysis would point out new biomarkers and/or combination of biomarkers that could help in early diagnoses of asymptomatic patients, and can be for differential diagnosis and/or prognostic marker for AD and DLB **(Figure 19)**.

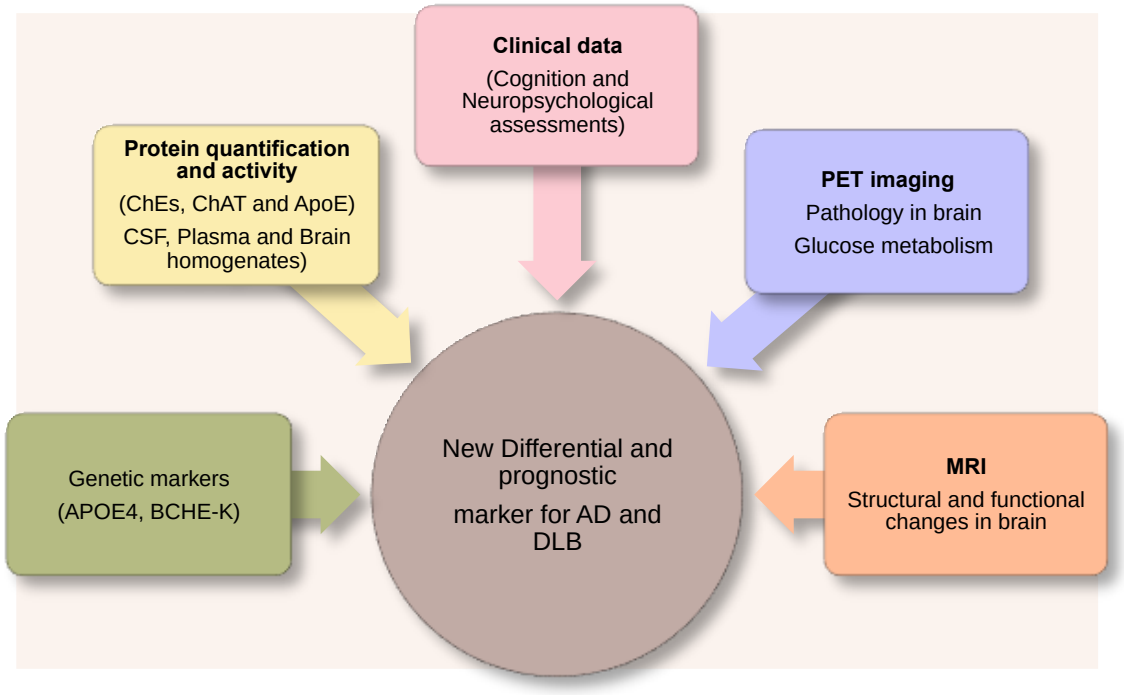

Figure 19. Schematic presentation of the possible multivariate analysis for a new differential and prognostic marker for AD and DLB

APOE4 is the main confirmed genetic risk factor for sporadic AD, an understanding of the molecular mechanism concerning its abnormal levels in the brain, CSF and plasma, perhaps along with BuChE activity and protein levels may be fundamental and instrumental in the understanding the disease mechanism and development of effective therapeutic agents.

Considering that, despite over three decades of intensive search for new therapeutic agents, and numerous failed clinical trials on agents targeting APP metabolism, Aβ aggregation, and deposition, the ChE Inhibitors are still the main therapeutic options for the symptomatic

treatment in AD, DLB and PDD, the proposed molecular interaction of ApoE with BuChE and Aβ might prove to be a more fruitful therapeutic target, in particular since it brings together several plausible and/or well documented phenomenon, such as dysfunctional Aβ production, accumulation and clearance, selective vulnerability of cholinergic neuronal networks and neuroinflammatory processes involving astroglial cells function that may be under regulation of ACh signaling.

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9 REFERENCES

1. Manyam, B.V., *Dementia in Ayurveda.* J Altern Complement Med, 1999. **5**(1): p. 81-8.
2. Boller, F. and M.M. Forbes, *History of dementia and dementia in history: an overview.* J Neurol Sci, 1998. **158**(2): p. 125-33.
3. Berchtold, N.C. and C.W. Cotman, *Evolution in the Conceptualization of Dementia and Alzheimer's Disease: Greco-Roman Period to the 1960s.* Neurobiology of Aging, 1998. **19**(3): p. 173-189.
4. Alzheimer, A., et al., *An English translation of Alzheimer's 1907 paper, "Uber eine eigenartige Erkankung der Hirnrinde".* Clin Anat, 1995. **8**(6): p. 429-31.
5. Alzheimer's, A., *2016 Alzheimer's disease facts and figures.* Alzheimers Dement, 2016. **12**(4): p. 459-509.
6. Korolev, I.O., *Alzheimer's Disease: A Clinical and Basic Science Review.* Medical Student Research Journal, 2014. **4**: p. 24-33.
7. Tiraboschi, P., et al., *The importance of neuritic plaques and tangles to the development and evolution of AD.* Neurology, 2004. **62**(11): p. 1984-1989.
8. Bronzuoli, M.R., et al., *Targeting neuroinflammation in Alzheimer's disease.* Journal of Inflammation Research, 2016. **9**: p. 199.
9. Selkoe, D.J., *Alzheimer's disease is a synaptic failure.* Science, 2002. **298**(5594): p. 789-791.
10. Francis, P.T., et al., *The cholinergic hypothesis of Alzheimer's disease: a review of progress.* Journal of Neurology, Neurosurgery & Psychiatry, 1999. **66**(2): p. 137-147.
11. Ewers, M., et al., *Neuroimaging markers for the prediction and early diagnosis of Alzheimer's disease dementia.* Trends in neurosciences, 2011. **34**(8): p. 430-442.
12. Hamilton, R.L., *Lewy Bodies in Alzheimer's Disease: A Neuropathological Review of 145 Cases Using α-Synuclein Immunohistochemistry.* Brain pathology, 2000. **10**(3): p. 378-384.
13. Bachman, D.L., et al., *Prevalence of dementia and probable senile dementia of the Alzheimer type in the Framingham Study.* Neurology, 1992. **42**(1): p. 115-9.
14. Rademakers, R., M. Cruts, and C. Van Broeckhoven, *Genetics of early-onset Alzheimer dementia.* ScientificWorldJournal, 2003. **3**: p. 497-519.
15. Zekanowski, C., et al., *Genetic aspects of Alzheimer's disease.* Acta Neurobiol Exp (Wars), 2004. **64**(1): p. 19-31.
16. Tsai, M., et al., *Apolipoprotein E: risk factor for Alzheimer disease.* American journal of human genetics, 1994. **54**(4): p. 643.
17. Kosaka, K., et al., *Presenile dementia with Alzheimer-, Pick- and Lewy-body changes.* Acta Neuropathol, 1976. **36**(3): p. 221-33.
18. Fujishiro, H., et al., *Distribution of cerebral amyloid deposition and its relevance to clinical phenotype in Lewy body dementia.* Neurosci Lett, 2010. **486**(1): p. 19-23.
19. Marui, W., et al., *Pathological entity of dementia with Lewy bodies and its differentiation from Alzheimer's disease.* Acta Neuropathol, 2004. **108**(2): p. 121-8.
20. McKeith, I., *Dementia with Lewy bodies.* Handb Clin Neurol, 2007. **84**: p. 531-48.
21. Bliwise, D.L., et al., *Sleep disturbance in dementia with Lewy bodies and Alzheimer's disease: a multicenter analysis.* Dementia and geriatric cognitive disorders, 2011. **31**(3): p. 239-246.
22. McKeith, I.G., et al., *Consensus guidelines for the clinical and pathologic diagnosis of dementia with Lewy bodies (DLB): report of the consortium on DLB international workshop.* Neurology, 1996. **47**(5): p. 1113-24.

23. Aarsland, D., U.P. Mosimann, and I.G. McKeith, *Role of cholinesterase inhibitors in Parkinson's disease and dementia with Lewy bodies.* J Geriatr Psychiatry Neurol, 2004. **17**(3): p. 164-71.
24. Nelson, P.T., et al., *Association between male gender and cortical Lewy body pathology in large autopsy series.* Journal of neurology, 2010. **257**(11): p. 1875-1881.
25. Parkinson, J., *An essay on the shaking palsy.* The Journal of neuropsychiatry and clinical neurosciences, 2002. **14**(2): p. 223-236.
26. Lew, M., *Overview of Parkinson's Disease.* Pharmacotherapy: The Journal of Human Pharmacology and Drug Therapy, 2007. **27**(12P2): p. 155S-160S.
27. Padovani, A., et al., *Parkinson's disease and dementia.* Neurological Sciences, 2006. **27**(1): p. s40-s43.
28. Calne, D.B., *Treatment of Parkinson's disease.* New England Journal of Medicine, 1993. **329**(14): p. 1021-1027.
29. Titova, N., et al., *Parkinson's: a syndrome rather than a disease?* Journal of Neural Transmission, 2016: p. 1-8.
30. Lill, C.M., *Genetics of Parkinson's disease.* Molecular and Cellular Probes, 2016. **30**(6): p. 386-396.
31. Kivipelto, M., et al., *Risk score for the prediction of dementia risk in 20 years among middle aged people: a longitudinal, population-based study.* The Lancet Neurology, 2006. **5**(9): p. 735-741.
32. Frances, A., *Diagnostic and statistical manual of mental disorders: DSM-IV: prepared by the Task Force on DSM-IV*. 1994: American Psychiatric Assoc.
33. Association, A.P., *Diagnostic and statistical manual of mental disorders DSM-IV-TR fourth edition (text revision).* 2000.
34. Association, A.P., *DSM 5*. 2013: American Psychiatric Association.
35. Organization, W.H., *The ICD-10 classification of mental and behavioural disorders: diagnostic criteria for research.* 1993.
36. McKhann, G., et al., *Clinical diagnosis of Alzheimer's disease Report of the NINCDS-ADRDA Work Group* under the auspices of Department of Health and Human Services Task Force on Alzheimer's Disease.* Neurology, 1984. **34**(7): p. 939-939.
37. McKeith, I., et al., *Diagnosis and management of dementia with Lewy bodies third report of the DLB consortium.* Neurology, 2005. **65**(12): p. 1863-1872.
38. Emre, M., et al., *Clinical diagnostic criteria for dementia associated with Parkinson's disease.* Movement disorders, 2007. **22**(12): p. 1689-1707.
39. Postuma, R.B., et al., *MDS clinical diagnostic criteria for Parkinson's disease.* Movement Disorders, 2015. **30**(12): p. 1591-1601.
40. Fritz, N.E., et al., *Motor performance differentiates individuals with Lewy body dementia, Parkinson's and Alzheimer's disease.* Gait & Posture, 2016. **50**: p. 1-7.
41. Scharre, D.W., et al., *Paired Studies Comparing Clinical Profiles of Lewy Body Dementia with Alzheimer's and Parkinson's Diseases.* J Alzheimers Dis, 2016. **54**(3): p. 995-1004.
42. Noe, E., et al., *Comparison of dementia with Lewy bodies to Alzheimer's disease and Parkinson's disease with dementia.* Movement Disorders, 2004. **19**(1): p. 60-67.
43. Mandal, P.K., et al., *Interaction between Aβ Peptide and α Synuclein: Molecular Mechanisms in Overlapping Pathology of Alzheimer's and Parkinson's in Dementia with Lewy Body Disease.* Neurochemical Research, 2006. **31**(9): p. 1153-1162.
44. Jorm, A.F., *History of depression as a risk factor for dementia: an updated review.* Australian and New Zealand Journal of Psychiatry, 2001. **35**(6): p. 776-781.

45. Jackson, C.E., *Cholinergic System,* in *Encyclopedia of Clinical Neuropsychology,* J.S. Kreutzer, J. DeLuca, and B. Caplan, Editors. 2011, Springer New York: New York, NY. p. 562-564.
46. Tuček, S., *Regulation of acetylcholine synthesis in the brain.* Journal of neurochemistry, 1985. **44**(1): p. 11-24.
47. Jeger, R.V., *Mens sana in corpore sano revisited.* European heart journal, 2013: p. eht244.
48. Soreq, H. and S. Seidman, *Acetylcholinesterase—new roles for an old actor.* Nature Reviews Neuroscience, 2001. **2**(4): p. 294-302.
49. Nachmansohn, D. and A. Machado, *The formation of acetylcholine. A new enzyme:" Choline acetylase".* Journal of neurophysiology, 1943. **6**(5): p. 397-403.
50. Oda, Y., *Choline acetyltransferase: the structure, distribution and pathologic changes in the central nervous system.* Pathology international, 1999. **49**(11): p. 921-937.
51. Tooyama, I. and H. Kimura, *A protein encoded by an alternative splice variant of choline acetyltransferase mRNA is localized preferentially in peripheral nerve cells and fibers.* Journal of chemical neuroanatomy, 2000. **17**(4): p. 217-226.
52. Grosman, D.D., et al., *The human choline acetyltransferase gene encodes two proteins.* Journal of neurochemistry, 1995. **65**(2): p. 484-491.
53. Ozturk, A., S.T. DeKosky, and M.I. Kamboh, *Genetic variation in the choline acetyltransferase (CHAT) gene may be associated with the risk of Alzheimer's disease.* Neurobiology of aging, 2006. **27**(10): p. 1440-1444.
54. Grisaru, D., et al., *Structural roles of acetylcholinesterase variants in biology and pathology.* European Journal of Biochemistry, 1999. **264**(3): p. 672-686.
55. Bartels, C.F., T. Zelinski, and O. Lockridge, *Mutation at codon 322 in the human acetylcholinesterase (ACHE) gene accounts for YT blood group polymorphism.* American journal of human genetics, 1993. **52**(5): p. 928.
56. Darreh- Shori, T., et al., *Long- lasting acetylcholinesterase splice variations in anticholinesterase- treated Alzheimer's disease patients.* Journal of neurochemistry, 2004. **88**(5): p. 1102-1113.
57. Darvesh, S., D.A. Hopkins, and C. Geula, *Neurobiology of butyrylcholinesterase.* Nature Reviews Neuroscience, 2003. **4**(2): p. 131-138.
58. Darreh-Shori, T., et al., *Apolipoprotein epsilon4 modulates phenotype of butyrylcholinesterase in CSF of patients with Alzheimer's disease.* J Alzheimers Dis, 2012. **28**(2): p. 443-58.
59. Blokland, A., *Acetylcholine: a neurotransmitter for learning and memory?* Brain Research Reviews, 1995. **21**(3): p. 285-300.
60. Schliebs, R. and T. Arendt, *The cholinergic system in aging and neuronal degeneration.* Behavioural brain research, 2011. **221**(2): p. 555-563.
61. Pavlov, V.A., et al., *Brain acetylcholinesterase activity controls systemic cytokine levels through the cholinergic anti-inflammatory pathway.* Brain, behavior, and immunity, 2009. **23**(1): p. 41-45.
62. Tracey, K., *The inflammatory reflex Nature 420: 853–859.* Find this article online, 2002.
63. Parrish, W.R., et al., *Modulation of TNF release by choline requires alpha7 subunit nicotinic acetylcholine receptor-mediated signaling.* Mol Med, 2008. **14**(9-10): p. 567-74.
64. Holtzman, D.M., J. Herz, and G. Bu, *Apolipoprotein E and apolipoprotein E receptors: normal biology and roles in Alzheimer disease.* Cold Spring Harbor perspectives in medicine, 2012. **2**(3): p. a006312.
65. Huang, Y., *Aβ-independent roles of apolipoprotein E4 in the pathogenesis of Alzheimer's disease.* Trends in molecular medicine, 2010. **16**(6): p. 287-294.

66. Strittmatter, W.J. and A.D. Roses, *Apolipoprotein E and Alzheimer disease.* Proceedings of the National Academy of Sciences, 1995. **92**(11): p. 4725-4727.
67. Berge, G., et al., *Apolipoprotein E ε2 genotype delays onset of dementia with Lewy bodies in a Norwegian cohort.* Journal of Neurology, Neurosurgery & Psychiatry, 2014. **85**(11): p. 1227-1231.
68. Liu, C.-C., et al., *Apolipoprotein E and Alzheimer disease: risk, mechanisms and therapy.* Nature Reviews Neurology, 2013. **9**(2): p. 106-118.
69. Darreh-Shori, T., et al., *The apolipoprotein E ε4 allele plays pathological roles in AD through high protein expression and interaction with butyrylcholinesterase.* Neurobiology of aging, 2011. **32**(7): p. 1236-1248.
70. Darreh-Shori, T., et al., *Functional variability in butyrylcholinesterase activity regulates intrathecal cytokine and astroglial biomarker profiles in patients with Alzheimer's disease.* Neurobiology of aging, 2013. **34**(11): p. 2465-2481.
71. Herz, J. and U. Beffert, *Apolipoprotein E receptors: linking brain development and Alzheimer's disease.* Nature Reviews Neuroscience, 2000. **1**(1): p. 51-58.
72. Kumar, R., A. Nordberg, and T. Darreh-Shori, *Amyloid-beta peptides act as allosteric modulators of cholinergic signalling through formation of soluble BAbetaACs.* Brain, 2016. **139**(Pt 1): p. 174-92.
73. Chow, V.W., et al., *An overview of APP processing enzymes and products.* Neuromolecular medicine, 2010. **12**(1): p. 1-12.
74. Darreh-Shori, T., et al., *Differential levels of apolipoprotein E and butyrylcholinesterase show strong association with pathological signs of Alzheimer's disease in the brain in vivo.* Neurobiology of aging, 2011. **32**(12): p. 2320. e15-2320. e32.
75. Hardy, J. and D.J. Selkoe, *The amyloid hypothesis of Alzheimer's disease: progress and problems on the road to therapeutics.* Science, 2002. **297**(5580): p. 353-356.
76. Lucey, B.P. and R.J. Bateman, *Amyloid-beta diurnal pattern: possible role of sleep in Alzheimer's disease pathogenesis.* Neurobiol Aging, 2014. **35 Suppl 2**: p. S29-34.
77. Iqbal, K., et al., *Tau pathology in Alzheimer disease and other tauopathies.* Biochimica et Biophysica Acta (BBA)-Molecular Basis of Disease, 2005. **1739**(2): p. 198-210.
78. Tartaglia, M.C., H.J. Rosen, and B.L. Miller, *Neuroimaging in dementia.* Neurotherapeutics, 2011. **8**(1): p. 82-92.
79. Association, A.s. and M. Albert, *The use of MRI and PET for clinical diagnosis of dementia and investigation of cognitive impairment: a consensus report*. 2003: Alzheimer's Association.
80. Alsop, D.C., et al., *Arterial spin labeling blood flow MRI: its role in the early characterization of Alzheimer's disease.* Journal of Alzheimer's Disease, 2010. **20**(3): p. 871-880.
81. Risacher, S.L. and A.J. Saykin. *Neuroimaging biomarkers of neurodegenerative diseases and dementia*. in *Seminars in neurology*. 2013. Thieme Medical Publishers.
82. Gomperts, S., et al., *Imaging amyloid deposition in Lewy body diseases.* Neurology, 2008. **71**(12): p. 903-910.
83. de la Fuente-Fernández, R., *Role of DaTSCAN and clinical diagnosis in Parkinson disease.* Neurology, 2012. **78**(10): p. 696-701.
84. Brosch, J.R., et al., *Tau Imaging in Alzheimer's Disease Diagnosis and Clinical Trials.* Neurotherapeutics, 2016: p. 1-7.
85. Gulyás, B., et al., *Activated MAO-B in the brain of Alzheimer patients, demonstrated by [11 C]-L-deprenyl using whole hemisphere autoradiography.* Neurochemistry international, 2011. **58**(1): p. 60-68.
86. Roy, R., et al., *Cholinergic imaging in dementia spectrum disorders.* European journal of nuclear medicine and molecular imaging, 2016. **43**(7): p. 1376-1386.

87. Hughes, A.J., et al., *Accuracy of clinical diagnosis of idiopathic Parkinson's disease: a clinico-pathological study of 100 cases.* Journal of Neurology, Neurosurgery & Psychiatry, 1992. **55**(3): p. 181-184.
88. Hughes, A.J., S.E. Daniel, and A.J. Lees, *Improved accuracy of clinical diagnosis of Lewy body Parkinson's disease.* Neurology, 2001. **57**(8): p. 1497-1499.
89. Association, A.P. and A.P. Association, *Diagnostic and statistical manual of mental disorders (DSM).* Washington, DC: American psychiatric association, 1994: p. 143-7.
90. Vijayaraghavan, S., et al., *High apolipoprotein E in cerebrospinal fluid of patients with Lewy body disorders is associated with dementia.* Alzheimer's & Dementia, 2014. **10**(5): p. 530-540. e1.
91. McDonald, W.I., et al., *Recommended diagnostic criteria for multiple sclerosis: guidelines from the International Panel on the diagnosis of multiple sclerosis.* Annals of neurology, 2001. **50**(1): p. 121-127.
92. Kurtzke, J.F., *Rating neurologic impairment in multiple sclerosis an expanded disability status scale (EDSS).* Neurology, 1983. **33**(11): p. 1444-1444.
93. Aarsland, D., et al., *Frequency and case identification of dementia with Lewy bodies using the revised consensus criteria.* Dementia and geriatric cognitive disorders, 2008. **26**(5): p. 445-452.
94. Sando, S.B., et al., *APOE ε4 lowers age at onset and is a high risk factor for Alzheimer's disease; A case control study from central Norway.* BMC neurology, 2008. **8**(1): p. 9.
95. Mulugeta, E., et al., *CSF amyloid β38 as a novel diagnostic marker for dementia with Lewy bodies.* Journal of Neurology, Neurosurgery & Psychiatry, 2010: p. jnnp. 2009.199398.
96. Mulugeta, E., et al., *Cerebrospinal fluid levels of sAPPα and sAPPβ in Lewy body and Alzheimer's disease: clinical and neurochemical correlates.* International Journal of Alzheimer's Disease, 2011. **2011**.
97. Maetzler, W., et al., *Reduced but not oxidized cerebrospinal fluid glutathione levels are lowered in Lewy body diseases.* Movement Disorders, 2011. **26**(1): p. 176-181.
98. Darreh-Shori, T., et al., *Changes in the activity and protein levels of CSF acetylcholinesterases in relation to cognitive function of patients with mild Alzheimer's disease following chronic donepezil treatment.* Journal of neural transmission, 2006. **113**(11): p. 1791-1801.
99. Menzel, E., et al., *Chemiluminescence assay for choline acetyltransferase in tissue extracts by using immune adsorption on monoclonal antibody.* Analytica Chimica Acta, 1988. **205**: p. 183-193.
100. Behbahani, H., et al., *Association of Omi/HtrA2 with γ-secretase in mitochondria.* Neurochemistry international, 2010. **57**(6): p. 668-675.
101. Arai, H., et al., *Apolipoprotein E gene in Parkinson's disease with or without dementia.* The Lancet, 1994. **344**(8926): p. 889.
102. Singleton, A.B., et al., *Clinical and neuropathological correlates of apolipoprotein E genotype in dementia with Lewy bodies.* Dementia and geriatric cognitive disorders, 2002. **14**(4): p. 167-175.
103. Bu, G., *Apolipoprotein E and its receptors in Alzheimer's disease: pathways, pathogenesis and therapy.* Nature Reviews Neuroscience, 2009. **10**(5): p. 333-344.
104. Gong, J.-S., et al., *Apolipoprotein E (ApoE) isoform-dependent lipid release from astrocytes prepared from human ApoE3 and ApoE4 knock-in mice.* Journal of Biological Chemistry, 2002. **277**(33): p. 29919-29926.
105. Lindh, M., et al., *Cerebrospinal fluid apolipoprotein E (apoE) levels in Alzheimer's disease patients are increased at follow up and show a correlation with levels of tau protein.* Neuroscience letters, 1997. **229**(2): p. 85-88.

106. Shafaati, M., et al., *Levels of ApoE in cerebrospinal fluid are correlated with Tau and 24S-hydroxycholesterol in patients with cognitive disorders.* Neuroscience letters, 2007. **425**(2): p. 78-82.
107. Blennow, K., C. Hesse, and P. Fredman, *Cerebrospinal fluid apolipoprotein E is reduced in Alzheimer's disease.* Neuroreport, 1994. **5**(18): p. 2534-2536.
108. Hesse, C., et al., *Measurement of apolipoprotein E (apoE) in cerebrospinal fluid.* Neurochemical research, 2000. **25**(4): p. 511-517.
109. Riddell, D.R., et al., *Impact of apolipoprotein E (ApoE) polymorphism on brain ApoE levels.* Journal of Neuroscience, 2008. **28**(45): p. 11445-11453.
110. Bartels, C., et al., *DNA mutation associated with the human butyrylcholinesterase K-variant and its linkage to the atypical variant mutation and other polymorphic sites.* American journal of human genetics, 1992. **50**(5): p. 1086.
111. Lane, R.M. and Y. He, *Emerging hypotheses regarding the influences of butyrylcholinesterase-K variant, APOE ε4, and hyperhomocysteinemia in neurodegenerative dementias.* Medical hypotheses, 2009. **73**(2): p. 230-250.
112. Wiebusch, H., et al., *Further evidence for a synergistic association between APOEε4 and BCHE-K in confirmed Alzheimer's disease.* Human genetics, 1999. **104**(2): p. 158-163.
113. Raygani, A.V., et al., *Analysis of association between butyrylcholinesterase K variant and apolipoprotein E genotypes in Alzheimer's disease.* Neuroscience letters, 2004. **371**(2): p. 142-146.
114. Lane, R., et al., *Synergistic effect of apolipoprotein E ϵ4 and butyrylcholinesterase K-variant on progression from mild cognitive impairment to Alzheimer's disease.* Pharmacogenetics and genomics, 2008. **18**(4): p. 289-298.
115. Lehmann, D., C. Johnston, and A. Smith, *Synergy between the genes for butyrylcholinesterase K variant and apolipoprotein E4 in late-onset confirmed Alzheimer's disease.* Human molecular genetics, 1997. **6**(11): p. 1933-1936.
116. O'brien, K.K., et al., *Regulation of attention and response to therapy in dementia by butyrylcholinesterase.* Pharmacogenetics and Genomics, 2003. **13**(4): p. 231-239.
117. Holmes, C., et al., *Rate of progression of cognitive decline in Alzheimer's disease: effect of butyrylcholinesterase K gene variation.* Journal of Neurology, Neurosurgery & Psychiatry, 2005. **76**(5): p. 640-643.
118. Lane, R., et al., *BuChE- K and APOE ϵ4 allele frequencies in Lewy body dementias, and influence of genotype and hyperhomocysteinemia on cognitive decline.* Movement Disorders, 2009. **24**(3): p. 392-400.
119. Huang, Y.-W.A., et al., *ApoE2, ApoE3, and ApoE4 Differentially Stimulate APP Transcription and Aβ Secretion.* Cell, 2017.
120. Malmsten, L., et al., *Fibrillar β- amyloid 1- 42 alters cytokine secretion, cholinergic signalling and neuronal differentiation.* Journal of cellular and molecular medicine, 2014. **18**(9): p. 1874-1888.