

DISSERTATION

MULTIMODAL CHARACTERIZATION OF CANINE COGNITIVE DYSFUNCTION:
INTEGRATING NEUROPATHOLOGY, NEUROINFLAMMATION AND BIOMARKERS
TO MODEL ALZHEIMER'S DISEASE

Submitted by

Abdullatif Alsulami

Department of Environmental and Radiological Health Sciences

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Summer 2026

Doctoral Committee:

Advisor: Julie Moreno

Luke Montrose

Tara Nordgren

Tom LaRocca

Stephanie McGrath

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ABSTRACT

MULTIMODAL CHARACTERIZATION OF CANINE COGNITIVE DYSFUNCTION: INTEGRATING NEUROPATHOLOGY, NEUROINFLAMMATION AND BIOMARKERS TO MODEL ALZHEIMER'S DISEASE

Canine cognitive dysfunction syndrome (CCD) is a naturally occurring, age-associated neurodegenerative disorder in companion dogs that shares clinical and pathological features with Alzheimer's disease (AD), amyloid-beta ($A\beta$) deposition, tau-related pathology, and neuroinflammation. This dissertation aimed to characterize CCD as a translational model of AD by integrating fluid biomarkers, cognitive assessments, neuropathology, glial morphology, and spatial analysis.

First, biomarkers relevant to human AD, including neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), and $A\beta$, were assessed in aged dogs. Age and cognitive decline were associated with biomarker alterations, particularly increased NfL and reduced $A\beta_{1-42/1-40}$ ratios, supporting their potential use alongside behavioral assessment for CCD detection. Second, human AD neuropathological criteria were applied to aged canine brains. Dogs showed early to intermediate $A\beta$ deposition, limited regional tau pathology, and absence of neuritic plaques, supporting the canine model as a representation of early AD-like pathology.

In a larger cohort, cognitively impaired dogs showed higher $A\beta$ burden and more widespread glial activation compared with cognitively intact aged dogs. Multivariate analysis identified distinct pathological dimensions, including an amyloid-inflammatory

axis and tau-associated axes, while clustering revealed heterogeneous pathological subgroups. Finally, A β plaques relationship to glial cells were examined using morphological and spatial analyses. Astrocytes showed increased association with plaques, while microglial cells were more related to cognitive status

Overall, this dissertation demonstrates that CCD reflects a heterogeneous neurodegenerative syndrome that resembles critical features of AD, such as A β pathology, neurofibrillary tangles, biomarker changes, and prominent neuroinflammation. These findings support the companion dog as a valuable naturally aging translational model for studying AD-related cognitive decline, identifying biomarker signatures, and evaluating therapeutic strategies targeting amyloid pathology and neuroinflammation.

ACKNOWLEDGMENTS

I would like to express my deepest gratitude to my mother, whose unwavering support has been the foundation of everything I have accomplished. Your belief in me never changed, and I carry that with me in everything I do.

I also want to acknowledge my father. Although he did not get to see this moment, his influence continues to shape who I am today. I carry his values, his guidance, and his presence with me, and this achievement is as much his as it is mine.

To my wife, Lazaz, thank you for standing beside me through this journey. Your patience, encouragement, and constant support made this possible. You were there in every high and every low, and I could not have done this without you.

To my daughter, who came into my life just six months ago, you have already changed me in ways I did not know were possible. You gave this journey a deeper meaning and reminded me of what truly matters.

I would like to thank my advisor, Dr. Julie Moreno, for her mentorship, guidance, and support throughout my PhD. Your insight and direction shaped this work and helped me grow as a scientist. I am also thankful to my lab mates for their collaboration, support, and shared experiences, which made this journey productive and meaningful.

Finally, I am grateful to everyone who contributed to this work. This dissertation represents not only my effort, but the collective support of many great researchers who stood with me along the way.

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Chapter One: Introduction

1.1 Alzheimer's disease (AD) and related dementias

Neurodegenerative diseases comprise a diverse and heterogeneous group of progressive neurological disorders characterized by shared pathological characteristics, including selective neuronal vulnerability, synaptic dysfunction, and gradual loss of neural circuit integrity (Tesco & Lomoio, 2022). As global life expectancy continues to increase, these disorders are becoming a major public health concern, affecting tens of millions of individuals worldwide and placing substantial social and economic burdens on healthcare systems and caregivers. Among these disorders, Alzheimer's disease (AD) is the most prevalent cause of dementia and accounts for the majority of age-associated cognitive dysfunction. Epidemiological studies suggest that approximately 57 million people worldwide are living with dementia, a number expected to nearly triple by 2050 as populations continue to age (GBD 2019 Dementia Forecasting Collaborators, 2022). Despite decades of research, effective disease-modifying treatments remain limited, highlighting the need for improved understanding of disease mechanisms and more translational experimental models.

1.2 AD associated risk factors

Among the many risk factors associated with AD, aging remains the strongest and most consistent determinant of disease susceptibility (Guo et al., 2022). With increasing age, neurons go through progressive changes at the cellular level that reduce the brain's ability to maintain proteostasis, regulate inflammatory responses, and

preserve neuronal resilience (Coleman et al., 2026). Accumulated cellular damage, reduced efficiency of protein degradation systems, and altered mitochondrial function increase neuronal vulnerability to toxic protein aggregates and inflammatory stress. Furthermore, aging is associated with alterations in the central nervous system immune signaling, including dysregulated glial cells responses and increased inflammatory activity. Concurrently, cellular senescence contributes to the deterioration of structural and functional cellular integrity (García-Domínguez, 2025; Jiang et al., 2025). Together, these age-related changes create a biological environment that promotes protein aggregation and facilitates neurodegeneration.

Environmental and lifestyle factors also contribute greatly to the risk of AD. Epidemiological evidence increasingly links environmental exposures, including airborne pollutants, pesticides, heavy metals, and industrial chemicals, to elevated risk of cognitive decline and dementia (Adani et al., 2020; Dorsey & Bloem, 2024; Kilian & Kitazawa, 2018). More recently, exposure to wildfire smoke, as well as industrial contaminants such as per- and polyfluoroalkyl substances (PFAS) and microplastics, increase the risk of AD (Elser et al., 2025; Lu et al., 2024; Sahu et al., 2021; H. Yu et al., 2025). These environmental toxicants disrupt neuronal homeostasis, exacerbate oxidative stress, and promote inflammatory signaling pathways. In addition to environmental risk factors, genetic factors additionally play a critical role in disease susceptibility, particularly in familial forms of neurodegeneration. Mutations in genes such as amyloid precursor protein (APP), presenilin-1 (PSEN1), and presenilin-2 (PSEN2) are associated with early-onset familial AD (Borchelt et al., 1997).

Taken together, these observations suggest that AD arises from complex interactions among several domains, including aging, genetic susceptibility, environmental exposures, and cellular responses to protein aggregation. Understanding how these factors converge to drive neuronal dysfunction and cognitive decline remains a central challenge. Addressing this challenge requires models that capture the multifactorial and age-dependent nature of neurodegeneration, including the dynamic interplay between protein pathology and neuroinflammation.

1.3 Neuropathological features associated with AD

AD is neuropathologically defined by the accumulation of extracellular amyloid- β ($A\beta$) plaques and intracellular neurofibrillary tangles composed of hyperphosphorylated tau protein. These pathological lesions, collectively referred to as Alzheimer's disease neuropathologic change (ADNC), are accompanied by synaptic loss, neuronal degeneration, and progressive brain atrophy that ultimately leads to cognitive impairment. $A\beta$ pathology originates from the proteolytic processing of the amyloid precursor protein (APP). Under normal conditions, APP is cleaved by α -secretase, generating non-amyloidogenic fragments. In contrast, sequential cleavage by β -secretase and γ -secretase produces amyloid- β peptides, most commonly $A\beta_{40}$ and $A\beta_{42}$. Among these species, $A\beta_{42}$ is particularly prone to aggregation and forms oligomers and fibrillar deposits that accumulate in extracellular plaques.

Neuropathological diagnosis of AD is typically established through postmortem examination using standardized criteria. The National Institute on Aging-Alzheimer's Association (NIA-AA) guidelines integrate Thal phases of amyloid deposition, Braak

staging of tau pathology, and the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) scoring of neuritic plaque density to classify the degree of neuropathologic changes (Montine et al., 2012). The staging paradigm provides a structured framework for evaluating the presence, distribution, and severity of AD-associated lesions across the brain. Thal's staging captures the anatomical spread of A β deposition, Braak's staging reflects distribution of neurofibrillary tau pathology, and CERAD scoring provides an estimate of neuritic plaque burden. These measures combined classify ADNC into low, intermediate, and severe pathology.

The spatial distribution of amyloid deposition follows a relatively predictable pattern across brain regions. Early A β accumulation originates primarily within neocortical regions before spreading to limbic and subcortical structures. This pathological hierarchical progression is described by the Thal staging system (Thal et al., 2002), which outlines sequential phases of amyloid deposition. In addition to regional distribution, the burden of neuritic plaques is assessed using CERAD, which provide a semi-quantitative approach to evaluate the density of neuritic plaques in affected cortical regions. (Mirra et al., 1991). Tau pathology represents another major hallmark of AD. The distribution of tau pathology follows a characteristic anatomical progression described by the Braak staging system, beginning in the trans-entorhinal cortex and hippocampus before spreading to association cortices (Braak & Braak, 1991).

1.4 Pathological progression of misfolded proteins

Spatiotemporal progression of pathological proteins in AD reveals characteristics that explain how these proteins influence pathology in AD. A β is believed to be the initial insult, originating in association cortices, that initiates a cascade involving the spread of phosphorylated tau (van der Kant et al., 2020). This phenomenon is particularly evident when compared with patients with primary age-related tauopathy (PART), where phosphorylated tau distribution is restricted to the limbic system without any detectable deposition of A β . In contrast, in diseases where the two pathological proteins are present, A β facilitates the neocortical spread of tau, a pattern not observed in PART patients. This highlights the role of A β in promoting tau pathology spread and further exacerbating neurodegeneration. Unlike amyloid deposition, which may be observed decades prior to cognitive dysfunction, tau pathology is more strongly associated with neuronal loss and cognitive abnormalities (Rajmohan & Reddy, 2017; Tracy & Gan, 2018). Hyperphosphorylated tau disrupts cytoskeletal stability, interferes with axonal transport, and contributes to synaptic dysfunction.

1.5 molecular features of pathological protein aggregates

The accumulation of misfolded proteins represents a central molecular feature that is common in many neurodegenerative diseases. Under normal physiological conditions, newly synthesized proteins fold into specific conformations that enable them to perform defined cellular functions (Koszła & Sołek, 2024). Maintaining these structures requires a tightly regulated network of molecular chaperones and degradation pathways that ensure proper protein folding and remove damaged or misfolded

proteins. When these quality-control systems become disrupted, proteins may adopt abnormal conformations that promote aggregation and interfere with cellular function. Several major neurodegenerative diseases are associated with the aggregation of specific pathological proteins.

At the molecular level, protein aggregation typically involves conformational changes that shift proteins from their native structure toward β -sheet-rich assemblies capable of forming ordered fibrillar aggregates known as amyloids (Stefani & Rigacci, 2013). These fibrils are stabilized by hydrogen bonding and hydrophobic interactions between misfolded protein monomers. Once a misfolded conformation is established, aggregated proteins can act as templates that induce normally folded proteins to adopt similar abnormal structures. This process, commonly referred to as templated misfolding or seeding, allows aggregates to propagate and expand the reserve of pathogenic protein species (Prusiner, 2013). Early studies of neurodegenerative diseases focused primarily on large fibrillar deposits visible in postmortem tissue, such as amyloid plaques and neurofibrillary tangles, as these deposits were thought to represent the initial neurotoxic structures. However, a growing evidence suggests that smaller soluble oligomeric intermediates may represent the most neurotoxic forms of aggregated proteins (Ahmed et al., 2010; Rinauro et al., 2024; Vivoli Vega et al., 2019). These oligomers can disrupt synaptic signaling, alter membrane integrity, and interfere with intracellular trafficking prior to the formation fibrillar aggregates.

Many misfolded proteins associated with neurodegenerative diseases, including AD, have prion-like properties, such as the ability to self-propagate and spread between cells. Experimental studies demonstrate that aggregates of A β and phosphorylated tau

can seed pathology when inoculated into transgenic animals or cellular models, inducing corresponding pathology in recipient models (Clavaguera et al., 2013; Mougenot et al., 2012; Ruiz-Riquelme et al., 2018). This phenomenon provides a potential explanation for the stereotypical anatomical progression observed in several neurodegenerative diseases, where pathology spreads through interconnected neural networks.

1.6 Defense mechanisms against pathological proteins

Cellular protective mechanisms are designed to prevent the accumulation of misfolded proteins. The ubiquitin proteasome system represents one of the most important defense mechanisms that protects cellular structures from damaged or misfolded proteins. Through a cascade of enzymes (E1 activation, E2 conjugation, and E3 ligase enzymes) damaged proteins are tagged with ubiquitin molecules, marking them for proteasomal degradation (Ciechanover, 2013). Another defense pathway is chaperone-mediated autophagy (CMA) which is selective to misfolded proteins containing the KFERQ motif for lysosomal degradation (Y.-T. Wang & Lu, 2022). Misfolded proteins that escape the surveillance of these systems form aggregates and are subsequently degraded through macro-autophagy. Despite the presence of effective degradation machinery, certain factors such as aging, influence their ability to degrade and remove toxic protein aggregates.

Age-related impairment of proteostasis therefore plays a key role in the development of AD. As degradation pathways become less efficient, misfolded proteins accumulate and can overwhelm cellular quality-control systems (Hipp & Hartl, 2024;

Höhn et al., 2020). Once aggregation begins, these proteins can further disrupt intracellular trafficking and protein degradation pathways, creating a reinforcing cycle that promotes progressive neuronal injury. Emerging studies suggest that age-related proteostasis impairment is not limited solely to protein degradation networks, but involves broader failures in maintenance of proteome (Llewellyn et al., 2024). One prominent feature of aging is weakened relationship between transcriptomics and proteomic regulations, such that changes in gene expression doesn't predict changes in protein abundance or function (D. Wang et al., 2019). This age associated loss of transcriptome-proteome coupling suggests an increasingly disrupted proteostasis at post-transcriptional and translational levels, specifically in protein aggregation prone environments (Dick et al., 2023). In addition, the capacity of protein translation declines with aging, contributing further to impaired proteome maintenance. Across multiple species, aging has been shown to be associated with altered ribosomal function, ribosomal pausing, and increased mistranslation (Gerashchenko et al., 2021; Stein et al., 2022; Stein & Frydman, 2019). These age-related defects in proteostasis explain the cellular and functional deficits seen in NDs, with higher accumulation of misfolded proteins and dysfunctional protein degrading machineries.

Importantly, misfolded proteins do not act in isolation within the brain. Their interactions with surrounding cells, particularly glial cells, greatly influence their toxicity, clearance, and spread. Consequently, understanding neurodegeneration requires considering not only protein aggregation itself but also the broader cellular environment in which these aggregates develop.

1.7 Neuroinflammation is an integral component of AD pathology

Beyond amyloid and tau pathologies, AD involves widespread alterations in neuronal and glial function. Synapse loss represents one of the strongest pathological correlates of cognitive decline. Synaptic degeneration affects communication between vulnerable brain regions, particularly the hippocampus and association cortices, which are essential for memory, learning, and higher-order cognitive processing. Additional cellular processes, including mitochondrial dysfunction, oxidative stress, and altered metabolic signaling contribute to neuronal vulnerability during disease progression. These neuronal stress responses occur within an interactive environment, where injured or stressed neurons engage surrounding glial cells and activate immune signaling pathways. Eventually, neuronal injury and glial activation initiate an inflammatory cascade in the aging brain.

Neuroinflammation is increasingly recognized as a major component of AD pathogenesis (Deng et al., 2024). Although early research focused primarily on protein aggregation, increasing evidence demonstrates that inflammatory processes within the central nervous system play critical roles in shaping disease progression (McKenzie et al., 2025). These responses involve activation of glial cells, alterations in cytokine signaling, and recruitment of immune pathways that influence neuronal survival and protein clearance. Therefore, neuroinflammation is now believed to be an active response, rather than a secondary process, that can influence disease progression.

Under physiological conditions, immune homeostasis in the brain is maintained primarily by astrocytes and microglia (Hashimoto et al., 2025). These glial cells perform

essential functions including synaptic regulation, metabolic support, and clearance of cellular debris. Microglia act as resident immune cells of the central nervous system, while astrocytes regulate neurotransmitter balance, maintain extracellular ion concentrations, and contribute to blood–brain barrier integrity. Both cells are essential supporters of neuronal activity and resilient to changes in the surrounding microenvironment.

In AD, glial cells undergo substantial functional and morphological changes. Astrocytes and microglia become activated in response to pathological stimuli such as aggregated proteins and neuronal injury (J. Han et al., 2025). These cells release pro-inflammatory mediators that influence the surrounding environment. While early inflammatory responses may facilitate the removal of aggregated proteins and promote tissue repair, persistent activation can lead to chronic inflammation that disrupts neuronal function. This prolonged activation impairs the ability of glial cells to effectively clear toxic protein aggregates, which eventually shifts responses from protective to active contributors to disease progression.

Recent studies reveal that glial responses during neurodegeneration are highly heterogeneous (Casali et al., 2020; Serrano-Pozo et al., 2024). Rather than existing in a single reactive state, astrocytes and microglia adopt a range of functional phenotypes depending on disease stage and local microenvironment. Some glial states may promote neuroprotection and tissue repair, whereas others contribute to inflammation and neuronal damage. Genetic studies further support the role of immune pathways in AD. Genome-wide association studies (GWAS) have identified risk variants in genes involved in microglial function and inflammatory regulation, including triggering receptor

expressed on myeloid cells 2 (TREM2), which is associated with increased disease risk, indicating that dysregulated immune responses may directly influence disease susceptibility and progression (Guerreiro et al., 2013; Lambert et al., 2013).

Protein aggregates develop within a complex cellular environment where interactions with surrounding cells influence their formation, clearance, and toxicity (J. Wu et al., 2024). Among these cellular populations, glial cells play particularly important roles in shaping the progression of neurodegenerative diseases. In AD, astrocytes and microglia frequently accumulate around amyloid plaques, forming a localized microenvironment that surrounds sites of protein deposition (Y. Wu & Eisel, 2023). These cells respond to aggregated proteins through activation of pattern-recognition receptors and inflammatory signaling pathways. Initially, glial responses may support protective processes by promoting the phagocytosis and clearance of protein aggregates. Microglia can internalize A β through receptor-mediated pathways, while astrocytes contribute to maintaining extracellular homeostasis and regulating protein degradation mechanisms (Choi & Chung, 2025). However, prolonged exposure to aggregated proteins can lead to dysfunctional glial responses. Chronic activation is associated with increased inflammatory signaling, oxidative stress, and impaired protein clearance. In this context, glial cells may transition from protective responders to contributors of disease progression.

Glial cells also influence the propagation of pathological proteins. Microglia can internalize tau aggregates and release them in forms capable of seeding additional pathology, potentially facilitating the spread of tau across neural networks (C. Wang et al., 2022). Astrocytes may similarly accumulate misfolded proteins and undergo

functional changes that alter neuronal metabolism and inflammatory signaling (Katsouri et al., 2020; Muwanigwa et al., 2024).

The spatial organization of glial responses around protein aggregates is increasingly recognized as an important determinant of disease progression. Advances in spatial transcriptomics and imaging have revealed that glial cells located near pathological deposits exhibit distinct gene expression patterns associated with inflammatory signaling, metabolism, and proteostasis (W.-T. Chen et al., 2020; Marsh et al., 2022; Serrano-Pozo, Mielke, et al., 2011a).

These observations have shifted the conceptual framework of AD from protein-focused models toward integrated models that incorporate cellular context. Understanding how glial responses interact with pathological proteins is therefore essential for elucidating the mechanisms underlying neurodegeneration.

1.8 Diagnostic biomarkers in AD

The emergence of biomarkers in AD has provided critical insights into the prediction of underlying early pathological changes (Kwon et al., 2024). It has enabled the identification of disease processes in vivo, often years prior to the onset of clinical symptoms (Ausó et al., 2020). Within current frameworks, biomarkers are broadly categorized into those reflecting underlying pathology and neuronal injury. Neurofilament light chain (NfL) is a cytoskeletal protein that is associated with increasing axonal damage. Several studies have shown that circulating NfL levels in the CSF and plasma are associated with AD and other neurodegenerative diseases (Dittrich et al., 2022; Nakamura et al., 2024). However, NfL levels reflect non-specific neuronal

damage, which suggests that its use as a standalone biomarker is not specific for AD. Other detectable markers include A β isoforms (Gallingani et al., 2024). The ratio of A β isoforms, particularly A β_{42} /A β_{40} , corresponds to underlying amyloid pathology burden and can be detected in both CSF and plasma (Janelidze et al., 2016; Pérez-Grijalba et al., 2019).

In addition to these markers, glial-associated biomarkers such as glial fibrillary acidic protein (GFAP) have emerged as sensitive indicators of astrocytic activation and general neuroinflammation. In neurodegeneration, GFAP levels in the plasma and CSF provide insights into the underlying neuroinflammation (Leipp et al., 2024). Since astrocytes shift to a more reactive phenotype in response to A β deposition, higher GFAP levels may reflect early neuroinflammatory responses prior to neuronal degeneration (Bandara et al., 2025). Similar to NfL, GFAP is not a specific AD marker and should be integrated alongside other markers that reflect underlying pathology and neuronal damage.

Biomarkers provide stronger predictive value when they are used together, as each marker reflects a different aspect of AD pathology and disease progression. Circulating and cerebrospinal fluid levels of A β , phosphorylated tau, GFAP, and NfL have been shown to be associated with disease progression and pathological burden, reflecting key AD underlying processes including protein aggregation, neuronal injury, and gliosis (Pan et al., 2025). . Together, these biomarkers provide a more comprehensive assessment of disease status than any single marker alone. This highlights the importance of using combined biomarker panels to improve detection, monitor disease progression, and capture the complex biological changes associated

with AD. Recent advances in plasma-based biomarkers research have allowed for minimally invasive detection of AD-related underlying pathology, supporting their growing role in early diagnosis and disease progression monitoring (Heo et al., 2025).

1.9 The current state of animal models in AD research

Animal models have been essential for advancing our understanding of neurodegenerative disease mechanisms. Transgenic rodent models have provided valuable insights into AD pathology; however, many rely on overexpression of human genes associated with AD. As a result, these models do not fully reproduce the spontaneous and age-dependent nature of late-onset AD (Dawson et al., 2018; Singhaarachchi et al., 2025).

The companion dog has emerged as a valuable translational model because it naturally develops cognitive decline and neuropathological changes during aging (McGrath et al., 2025). Dogs share environmental exposures with humans, including diet and lifestyle factors, and possess substantial genetic diversity compared with laboratory animals. Aging dogs develop A β deposition in regions associated with cognition. These deposits resemble human diffuse plaques and increase with age (Mesquita et al., 2021; Pugliese et al., 2006; Shimada et al., 1992). Importantly, amyloid accumulation in dogs has been associated with behavioral changes and cognitive impairment. Although tau pathology has been considered less prominent in dogs, recent studies have identified tau pathology in aged canine brains (Abey et al., 2021a). These findings suggest that dogs may develop elements of both major Alzheimer's-related proteinopathies.

Behavioral manifestations in dogs include physical disturbances and cognitive alterations. Several dementia scales developed specifically for the dogs have provided valuable insights into association of behavioral symptoms in aged dogs. One of the scales is canine dementia scale (CADES), which contains 17 items distributed into four domains that are associated with dog's behavior. Spatial orientation, social interactions, sleep-wake cycles and house soiling represent the four domains used within CADES scale to identify various stage of cognitive dysfunction in aging dogs, including normal aging, mild cognitive impairment, moderate cognitive impairment and severe cognitive dysfunction (Madari et al., 2015). Another behavioral assessment that is used in assessing behavioral changes in aging dogs is the canine cognitive dysfunction rating (CCDR) scale (Salvin et al., 2011). CCDR survey quantifies behavioral changes associated with cognitive dysfunction, including alterations in memory, orientation, social interaction, and daily activities.

Because dogs develop age-associated cognitive decline naturally, they provide a unique opportunity to investigate relationships among neuropathology, cognitive status, and biological markers of disease progression in a translationally relevant context.

Despite extensive research, many fundamental aspects of Alzheimer's disease progression remain unresolved. While amyloid- β and tau pathology represent defining features of the disease, growing evidence indicates that interactions among protein aggregation, neuroinflammation, and cellular responses play critical roles in shaping disease trajectory. Traditional experimental models often fail to capture the spontaneous and multifactorial nature of late-onset neurodegeneration. Consequently, alternative models that reflect natural aging processes are needed to better understand disease

mechanisms. Another major gap involves the identification of reliable biomarkers capable of reflecting disease processes in living animals. In humans, biomarkers such as A β , phosphorylated tau, neurofilament light chain, and GFAP have proven useful for detecting and monitoring neurodegeneration. However, the diagnostic utility of these markers in dogs remains less well established.

Finally, the cellular microenvironment surrounding protein aggregates in the aging canine brain remains poorly characterized. Interactions between glial cells and pathological proteins may influence disease progression, yet these relationships have not been extensively investigated in canine models. These gaps in knowledge require integrated approaches combining behavioral assessments, neuropathology, biomarker analysis, and multivariate data analysis. Investigating these relationships in naturally aging dogs may provide important insights into the mechanisms underlying Alzheimer's-like neurodegeneration.

Chapter Two: Plasma and cerebrospinal fluid biomarkers in aged dogs with cognitive decline

Abdullatif Alsulami^{1,2}, Sean W. Boland^{1,2}, Stephanie McGrath^{2,3,4}, Evan L. MacLean⁵, Caitlin Latimer⁶, Martin Darvas⁶ and Julie A. Moreno^{1,2,4*}

1Department of Environmental & Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

2Brain Research Center, Colorado State University, Fort Collins, CO, USA

3Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

4Center for Healthy Aging, Colorado State University, Fort Collins, CO, USA

5Department of Psychology, College of Veterinary Medicine, University of Arizona, Tucson, USA

6Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, USA

2.1 Abstract

Background: Canine cognitive dysfunction syndrome (CCD) is a naturally occurring progressive neurodegenerative disease that commonly affects senior dogs, with age being the primary risk factor. Aging dogs present a valuable model for studying aging and neurodegeneration due to natural development of the disease and similarities to Alzheimer's disease (AD). In this study, we evaluated biomarkers that are relevant for human neurodegeneration, including neurofilament light chain (NfL), glial fibrillary acidic protein (GFAP), amyloid-beta and the amyloid-beta ($A\beta_{1-42/1-40}$) ratio, to explore the differences between healthy aging and CCD.

Results: Our results demonstrate significant associations between age and dementia biomarkers, with reduced $A\beta_{1-42/1-40}$ ratios in plasma, and elevated NfL levels in cerebrospinal fluid (CSF) at older ages. These biomarkers were also associated cognitive impairment, as assessed by owner-directed CCD surveys. Notably, NfL levels in plasma showed a strong positive correlation with both age and cognitive decline, suggesting its potential utility as a non-invasive diagnostic tool for CCD. While plasma NfL is promising, it is non-specific and can also be elevated due to other neurological conditions. Therefore, combining NfL with other biomarkers, such as GFAP and $A\beta$, alongside clinical assessments, may enable a more accurate diagnosis of CCD.

Conclusion: Our findings further support the use of dogs with CCD as a model for studying AD biomarkers, with implications for the development of therapeutic interventions in both dogs and humans.

2.2 Background

Senior dogs show clinical signs of cognitive decline as part of a syndrome called canine cognitive dysfunction (CCD), which shares remarkable similarities with Alzheimer's disease (AD) (Madari et al., 2015; Salvin et al., 2011). According to the American Veterinary Medical Foundation, the number of companion dogs over the age of 10 years has grown by 15% in the past 20 years. With the steadily increasing canine population, continued research into cognitive decline in aging dogs is critical, particularly given the implications and translational value for human aging and neurodegeneration. The prevalence of CCD is estimated to range from 14-35% of the senior canine population, with the incidence increasing exponentially with age (Chapagain et al., 2018; Salvin et al., 2010). In a study of 180 geriatric dogs (aged 11-16 years), 28% of dogs 11-12 years old and 68% of dogs 15-16 years old were found to exhibit signs of cognitive impairment (Azkona et al., 2009). In a two-year longitudinal study of dogs over the age of 8 years, 33% of dogs with normal cognitive status progressed to mild cognitive impairment, and 22% of dogs with mild impairment progressed to CCD (Dewey et al., 2021).

Pathologically, CCD is characterized by the loss of synaptic proteins, and the accumulation of misfolded proteins, specifically amyloid-beta ($A\beta$) and phosphorylated tau (P-tau), in the brains of affected dogs (Hines et al., 2023; Land et al., 2021). $A\beta$ deposits have been observed in aging dog brains, with the amyloid load often correlating with CCD signs (Head et al., 2010). P-tau protein has also been detected in the aging dog brain, though with less frequency and density than in human AD brains (Abey et al., 2021b; Smolek et al., 2016). Given the shared pathological characteristics,

CCD has emerged as a naturally occurring surrogate for the development of diagnostic tools and therapeutic interventions for human AD (Prpar Mihevc & Majdič, 2019a), offering a valuable model due to its analogous histopathological, behavioral, and cognitive alterations.

In humans, biomarkers for the diagnosis of AD have been extensively studied (Bateman et al., 2012; Blennow & Zetterberg, 2018). These include A β , P-tau, neurofilament light chain (NfL), and glial fibrillary acidic protein (GFAP), which are measurable in both blood and cerebrospinal fluid (CSF). A β reflects underlying amyloid plaque burden, P-tau indicates tau tangle formation, NfL marks axonal degeneration, and GFAP represents astrocyte activation and general neuroinflammation. Our data show that we can successfully detect A β , GFAP, and NfL in plasma and CSF derived from young and senior dogs and that these biomarkers correlate with cognitive status, suggesting their utility in diagnosing CCD, similar to AD.

2.3 Methodology

Enrollment criteria. Dogs were recruited through social media platforms and local news sources, a local network of family veterinarians to help identify dogs with signs of CCD at their practices, and the current pet owner population of senior dogs at the Veterinary Teaching Hospital Neurology Clinical Trials Service. All owners signed an informed consent as part of the enrollment process into study. The samples are listed in Table 1. Experiments performed were approved by the Institutional Animal Care and Use Committee at Colorado State University (Protocol #: 4106 and 1235).

Moreno ID	Name	VTH ID	CADES	CCDR		Age	Sex	Weight
Ca501	Radar Ricciadri	1023998	0	35	3.2	MC		9.9
Ca502	Gamble Standford	1017612	0	34	1.6	MC		27.3
Ca505	Loki George	1007849	0	33	2.5	MC		39.1
Ca506	Juniper Kallin	356899	0	NA	3.7	FS		21.7
Ca507	Annie Carlaw	1019113	0	34	3.9	FS		5.4
Ca509	Lenny Lopez	1014397	0	32	1.2	MC		11.25
Ca511	Glitch Outlaw	1015106	0	34	1.8	MC		18.2
Ca521	Elliot Rockow	1048937	2	35	6.2	MC		26.5
Ca524	Mila Shanchez	1048404	6	39	3.6	FS		40.6
Ca526	Jingle Bell Irigoyen	1045259	4	40	6.25	FS		23.7
Ca529	Moose Olmstead	1020496	0	34	2.3	MC		25.2
Ca535	Enzo Bailey	1034735	0	0	3.3	MC		18.4
Ca537	Lucy Moreno	1032950	0	34	1.9	FS		19.3
Ca547	Teaghan Mulligan	1037331	0	34	1.8	FS		14.8
Ca553	Voodoo Roberts	1018765	0	NA	2.5	MI		29.4
Ca556	Bella Xiao-Kim	1049585	2	36	5.2	SF		25
Ca557	Chandler Isdale	1016778	0	34	6.7	MC		28.1
Ca504	Hazel Davis	269079	0	NA	10	SF		19.6
Ca510	Denali Becker	274263	0	NA	10.9	MC		27.9
Ca512	Chaos Quintero	343004	6	NA	9.8	MC		16.3
Ca513	Bear MacQuiddy	1015872	0	34	8.2	SF		25
Ca514	Jake Outlaw	100622	0	NA	12.5	MC		24.8
Ca516/259	Zola Jones	1016781	0	NA	10.4	SF		23.7
Ca517	Stormy Kusick	1014208	6	35	9.9	MC		7.95
Ca519	Gracie Hill	1011221	6	NA	8.4	SF		24
Ca520	Kate Kusick	1039786	0	34	7.1	SF		10.5
Ca527	Duke Sauer	1033355	4	NA	10.5	MC		39.9
Ca528	Avery Sauer	1033357	2	NA	10.5	SF		33.5
Ca539	Rubix Ayers	355770	2	34	8.2	MC		20
Ca540	Kovu Barbeitos	1002556	2	36	10	MC		40.7
Ca532	Annie Ayers	1049559	0	34	7.5	SF		16
Ca542	Bonnie Blake	314883	7	NA	13	SF		18.18
Ca548	Dante McNulty	1049334	6	37	10.2	MC		29.5
Ca549	Hoyu Kim	1037674	0	34	8.1	MC		44.4
Ca551	Leo Dolgin	1017577	0	41	9.1	MC		28.3
Ca558	Brisby Curtis	1007407	4	25	7.8	MC		27.1
Ca559	Timmy Bennet	1013587	0	25	10	MC		29
Ca560	Wolfgang Malinin	335191	0	34	14.5	MC		20

Ca503	Daytona Anseth	1015049	68	47	9.8	SF	26.1
Ca515	Zenny Kallin	1003825	58	NA	13.6	SF	9.3
Ca518	Treasure Murphy	274365	37	NA	13.2	MI	13.05
Ca522	Boomer Nehring	1016345	49	NA	13.1	MC	18
Ca523	Shire Ashton	281330	64	NA	15.4	FS	6.3
Ca541	Trout Florian	1046591	93	79	21	SF	20.9
Ca546	Johnny Lourenco	1034374	47	NA	12.5671233	MC	8.98
Ca550	Oscar Hart	1034356	81	58	14.8	MC	13.3
Ca554	Tucker Halusker	1048033	11	43	3.9	MC	28
Ca508	Chy Stanford	317772	34	34	9.5	FS	27.7
Ca530	Marty Markham	1049816	10	35	5.1	MC	31.8
Ca525	Dezi Marsh	1016180	21	31	6	SF	12.55

Table 1. Cognitive status and general information from canine plasma and CSF samples

CADES and CCDR owner surveys. The primary cognitive functional outcomes are based on two owner-based assessment surveys, the Canine Dementia Scale (CADES) and the Canine Cognitive Dysfunction Rating scale (CCDR) (Madari et al., 2015; Salvin et al., 2011). CADES is unique from CCDR in its ability to differentiate clinical stages of dementia (mild, moderate, or severe cognitive impairment).

Collection of CSF and plasma. Once enrolled the dogs underwent anesthesia for the collection of cerebrospinal fluid (CSF). Anesthesia was approved and performed by the CSU veterinary teaching hospital anesthesiology team. Anesthesia was determined by the anesthesiologist for each individual patient but commonly used delivery of methadone (0.1-0.5mg/kg) or butorphanol (0.1-0.5mg/kg) SQ/IM (for healthy energetic animals) the addition of acepromazine 0.01-0.02 mg/kg SQ/IM or dexmedetomidine 3-5 mcg/kg IM may have been utilized. Whole blood was collected, placed into EDTA blood

collection tubes and centrifuged to separate plasma. Samples were stored at -80°C until used for experimentation.

Meso Scale Discovery (MSD) method. Frozen dog cortex samples were homogenized in chilled RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 150mM sodium chloride, 50mM Tris hydrochloride, 0.5mM magnesium sulfate, Complete Mini protease inhibitor and phosphatase inhibitor cocktail- all from Sigma-Aldrich; St. Louis, MO) by sonication on ice (3x 10 pulses – separated by 5 minutes). Homogenates were centrifuged for 45 min at 21,000 × g and 4 °C. Supernatants containing RIPA-soluble proteins were aliquoted and transferred into a new storage tube. The remaining pellet was washed with RIPA buffer and centrifuged a second time 45 min at 21,000 × g and 4 °C. Supernatants from the RIPA wash were combined with the first RIPA fraction, aliquoted and stored at -80 °C until further analysis. The remaining pellet was resuspended in in chilled 5M guanidine hydrochloride (Gu-HCl) with Complete Mini protease inhibitor, sonicated on ice (1x 10 pulses), and centrifuged for 30 min at 21,000 × g and 4 °C. The supernatant (Gu-HCl fraction) was aliquoted, transferred into a new storage tube and stored at -80 °C until further analysis. Total protein content for RIPA and Gu-HCl fractions was determined using BCA assay kits (Pierce; Rockford, IL). For quantification of beta-amyloid, we used the MSD A β Peptide Panel kit (V-PLEX K15200G, Meso Scale Diagnostics, Rockville, MD), which is a multiplex kit for quantification of A β 40 and A β 42. We used 200 ng protein from the Gu-HCl fraction in duplicate for each sample and followed the manufacturer's instructions for performing the assays. Absolute concentrations for A β 40 and A β 42 were calculated using standards from the kit. For quantification of P-tau, we coated MSD bare plates (MSD

#L15XA-3 Meso Scale Diagnostics, Rockville, MD) with 40 ng per well of a monoclonal antibody for pSer396-tau (Clone PHF-13, BioLegend #829003, San Diego, CA) overnight at 4 °C, blocked unspecific binding sites with 5% bovine-serum albumin for 1 hour, incubated 2,000 ng protein from the RIPA fraction in duplicate for each sample for 1 hour, and detected pSer396-bound P-tau antigens with 50 ng per well of biotinylated total-tau antibodies (Clone TAU-12, BioLegend #806506, San Diego, CA) for 1 hour, and incubated antibody-antigen sandwiches with sulfo-tag streptavidin compound (Meso Scale Diagnostics, Rockville, MD) at a 1:1 molar ratio relative to biotinylated antibodies. Concentrations of P-tau were recorded as arbitrary units.

Neurology 4-Plex E Assay (N4PE). 250ml of plasma and 50ml of CSF was aliquoted into conical tubes and packaged for shipping to Quanterix Corporation in Billerica, MA 01821.

Statistical analysis.

This study analyzed baseline associations between dog age, symptoms of dementia (CADES, CDDR) and concentrations of NfL, GFAP, and Ab (in CSF and plasma). Dogs were categorized based on their CADES scores and age: young (< 8 years old), cognitively impaired (CCD+; CADES score > 8) and CCD- (CADES score < 8; > 8 years old). Prior to conducting group comparisons, normality was assessed using the Shapiro-Wilk test, and Levene's test was performed to check for homogeneity of variance. For group comparisons, two-sample t-tests were used where assumptions of normality and homogeneity were met. One-way ANOVA was used to compare more than two groups. Pearson's correlation was computed to assess the association between CADES and CDDR scores, and dog age and biomarker concentrations (NfL,

GFAP, and Ab). In addition, multiple linear regression analyses were conducted to evaluate the independent effect of independent variables (age and NfL levels in the plasma) on NfL levels in the CSF. All statistical analyses were performed using R software (version 4.3.1) and GraphPad Prism (version 10.1.2), with statistical significance set at $p < 0.05$ for all analyses.

2.4 Results

We measured $A\beta_{1-42}$ protein levels in frontal cortex of brain homogenates using MSD assay (Figure 1A). $A\beta_{1-42}$ levels were elevated in the cortex of senior dogs, consistent with age-associated beta-amyloid accumulation in dogs (Urfer et al., 2021). In contrast, phosphorylation of tau at S396 site did not differ significantly between younger and senior dogs (Figure 1B).

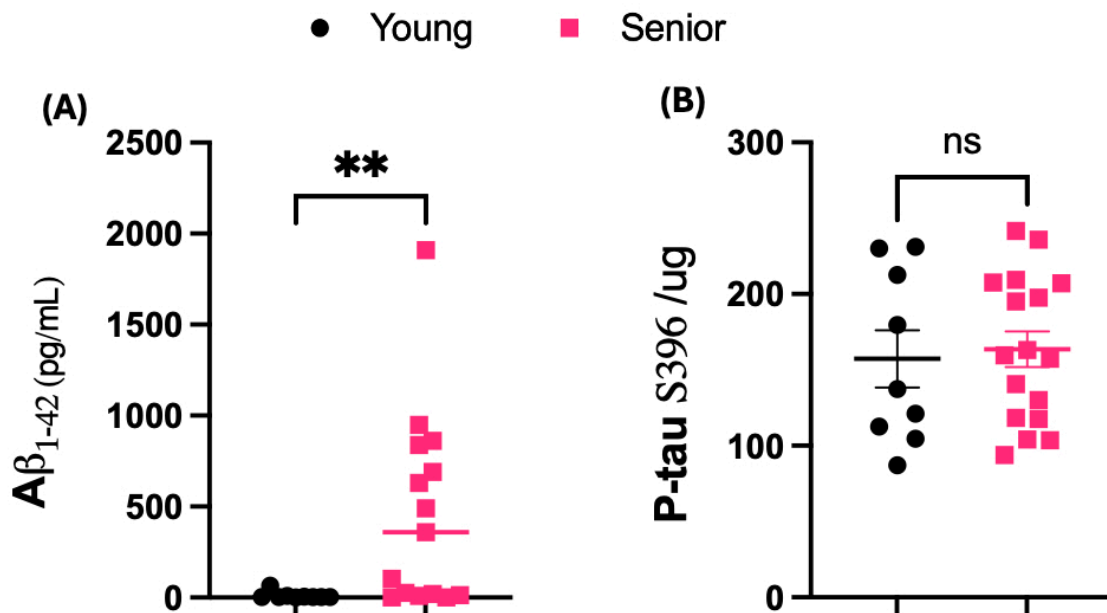


Figure 1. Protein levels of Amyloid b_{1-42} and phosphorylated tau (P-tau) in canine cerebral cortex samples grouped into two age categories. (A) Amyloid b_{1-42} levels are increased in dogs over the age of 9 years and (B) no change of P-tau S396 phosphorylation site was detected. N=25. (**p < 0.01, ns = non-significant).

In our cohorts of young, aged and aged dogs with cognitive dysfunction, we evaluated NfL, GFAP, $\text{A}\beta_{1-40}$, and $\text{A}\beta_{1-42}$ concentrations in both the CSF and plasma in aged and CCD+ dogs compared to young controls. NfL concentrations were increased in both the CSF and plasma of aged and aged demented dogs compared to the younger cohort. While the difference in CSF NfL was not statistically significant between aged and CCD+ dogs, plasma concentrations were significantly elevated in CCD+ dogs (Figure 2), suggesting that plasma NfL may serve as a marker of cognitive impairment. Similarly, GFAP concentrations, a known biomarker for neurodegeneration, were tending to increase in both CSF and plasma of CCD+ dogs, though statistical significance was not reached.

$\text{A}\beta$ levels in CSF and plasma are a widely used biomarker for AD (Lee et al., 2019). We measured both $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ isoforms in CSF and plasma, which provide more insights into amyloid pathology dynamics. $\text{A}\beta_{1-42}$ is more prone to aggregation and is a key component of amyloid plaques, whereas $\text{A}\beta_{1-40}$ is more abundant but less likely to aggregate. Separately, $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ levels did not differ significantly between CCD+ and CCD- dogs individually, the ratios are shown to be a more sensitive marker of amyloid burden. Separately, $\text{A}\beta_{1-40}$ and $\text{A}\beta_{1-42}$ levels did not differ significantly between young, aged, and aged with CCD dogs. However, their ratio ($\text{A}\beta_{1-42}/\text{A}\beta_{1-40}$) provided greater sensitivity to underlying amyloid burden. The CSF ratio of $\text{A}\beta_{1-42}/1-40$

was significantly lower in CCD+ dogs, indicating a more advanced A β burden (Figure 3). This finding is consistent with the accumulation of amyloid plaques, indicating a more advanced pathological state in these animals. In plasma, however, the ratio was not significantly different.

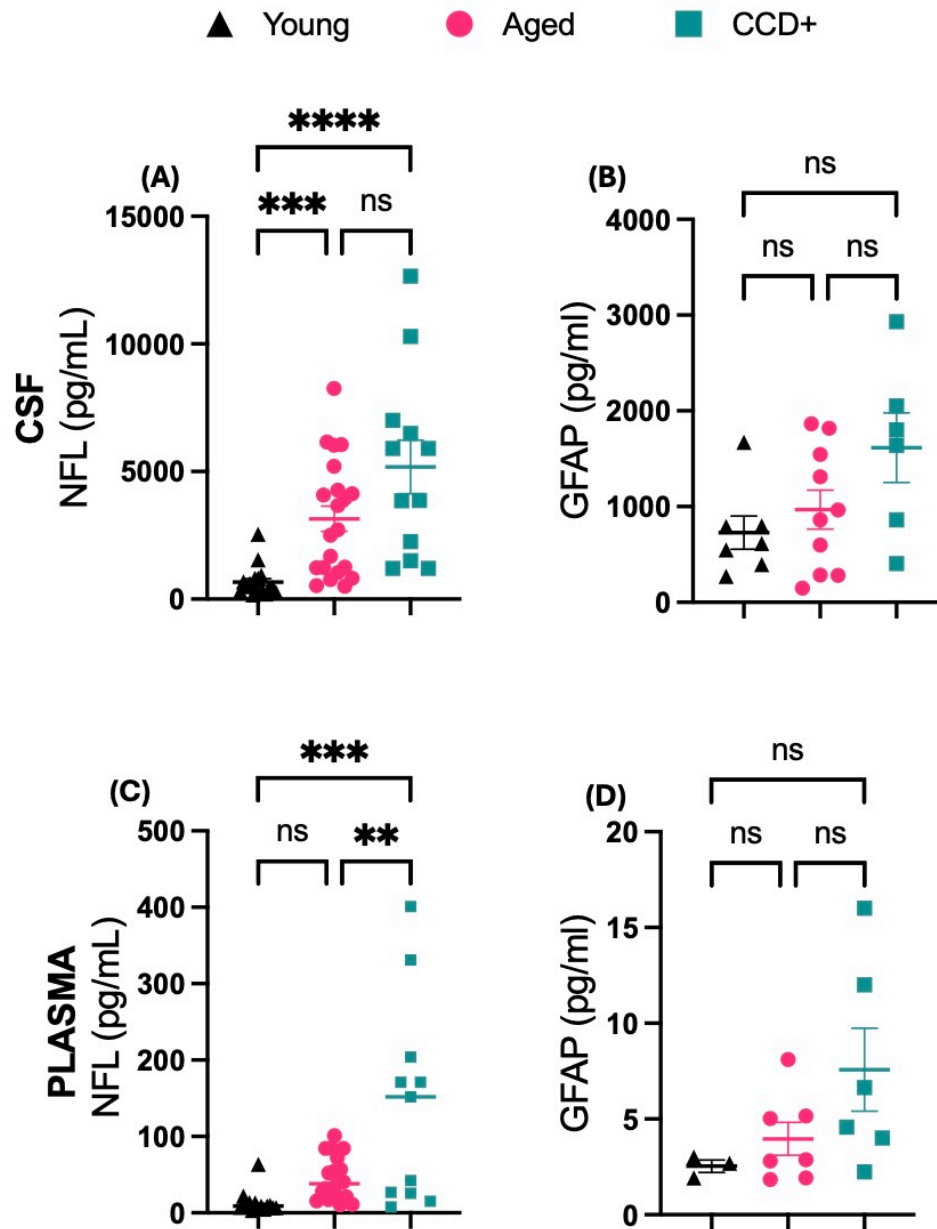


Figure 2. NFL and GFAP are elevated in CCD positive dogs. Levels of Neurofilament Light Chain (NFL) and Glial Fibrillary Acidic Protein (GFAP) in cerebrospinal fluid (CSF) and plasma between young and aged dogs with and without a diagnosis of canine cognitive dysfunction (CCD). The data was obtained using Quanterix Simoa assays. (* $p < 0.05$, ns = non-significant).

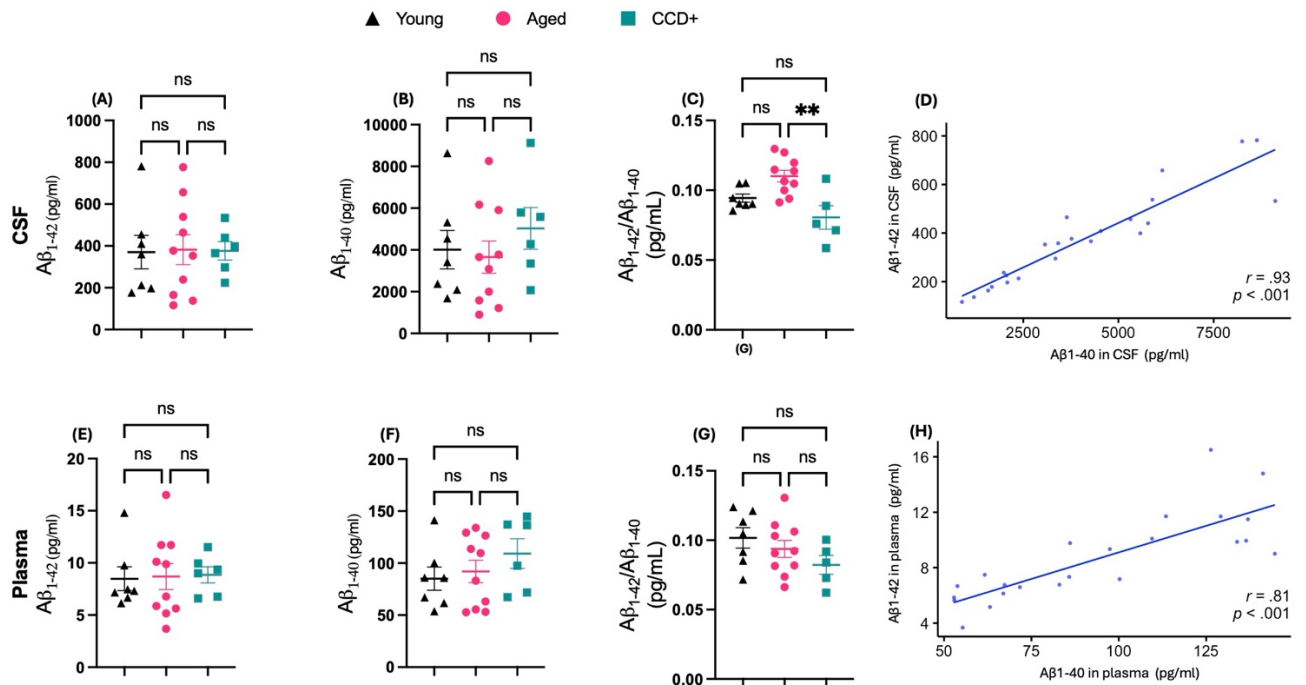


Figure 3. Amyloid b ratios decrease in CCD positive dogs. Concentrations of Amyloid-b ($A\beta_{1-42} / A\beta_{1-40}$) in cerebrospinal fluid (CSF) and plasma, presented as ratios between young and aged dogs with and without CCD. The ratios are analyzed using Quanterix Simoa assays to assess the correlation between these biomarkers and the presence of cognitive dysfunction. * $p < 0.05$, ** $p < 0.01$, ns = non-significant. (A) through (D) are $A\beta$ ratios in the CSF. (A) no significant changes in $A\beta$ 1-42 levels between young, aged & CCD+ dogs. (B) no significant $A\beta$ 1-40 ratio in CCD+ dogs. (D) significant correlation between $A\beta$ 1-42 and $A\beta$ 1-40 in CSF. (E) through (H) are $A\beta$ ratios in the plasma. (E)

no significant changes in A β 1-42 levels between young, aged & CCD+ dogs. (F) no significant changes in A β 1-40 levels between young, aged & CCD+ dogs. (G) no significant changes in A β 1-42/ A β 1-40 ratio in CCD+ dogs. (H) significant correlation between A β 1-42 and A β 1-40 in plasma. ** $p < 0.01$, ns = non-significant. N= 15 (A β 1-42 & A β 1-40 in CSF), 12 (A β 1-42 & A β 1-40 in plasma).

To further assess the biomarkers utility, we conducted a series of correlation analyses between biomarkers levels, NfL, GFAP, and clinical variables, including age, CADES and CCDR scores. These analyses were aimed at determining how well each biomarker reflects disease progression and cognitive decline. Biomarker levels from both the plasma and CSF, and their relationships with several critical clinical parameters were assessed with age and cognitive parameters, including CADES and CCDR scores. By examining these associations, we aimed to determine how well each biomarker reflects the progression of neurodegenerative changes, cognitive decline, and the overall severity of CCD. GFAP levels in the CSF showed a significant positive correlation with age ($p = 0.006$, $r = 0.55$, Figure 4), reflecting age-related neuroinflammation. However, GFAP was not associated with either CADES nor CCDR scores were significantly correlated with GFAP levels in the CSF, suggesting that GFAP may not directly capture cognitive impairment in CCD based on our sample size. Similarly, plasma GFAP levels did not show a significant correlation with age or dementia scores. However, further studies with larger sample sizes could help clarify its potential as a non-invasive biomarker.

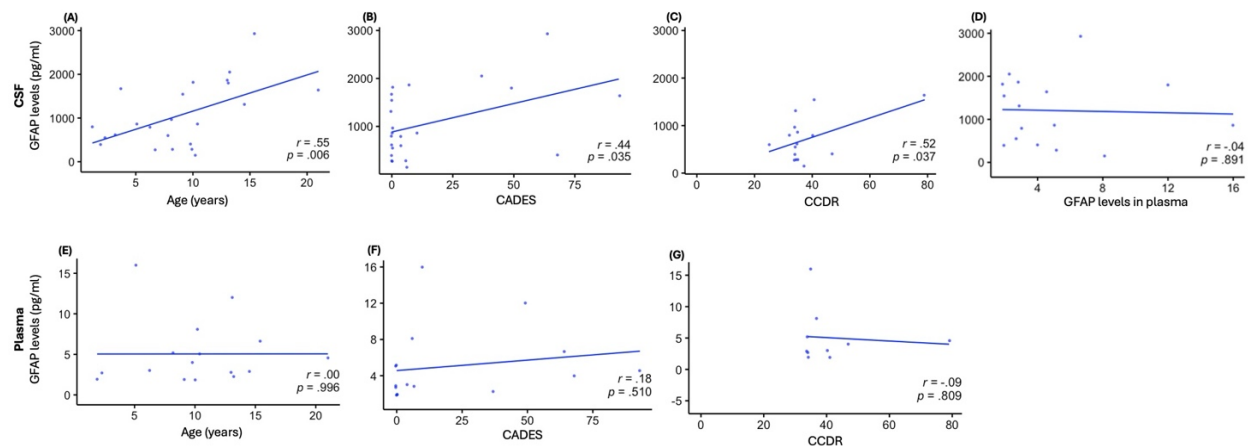


Figure 4. Correlation of GFAP Levels in CSF and Plasma vs. Age, CADES, CCDR and $A\beta_{1-42} / A\beta_{1-40}$ Ratios. Scatter plots showing the relationship between Glial Fibrillary Acidic Protein (GFAP) concentrations in cerebrospinal fluid (CSF) and plasma with age, CADES (Canine Dementia Scale), CCDR (Canine Cognitive Dysfunction Rating). These plots provide insights into how GFAP levels vary with different clinical and biological factors in aged dogs. (r = Pearson's coefficient, P = statistical significance value). (A) through (D) are GFAP values in the CSF plotted against age, CADES scores, CCDR scores, and GFAP values in the plasma (D). (A) there is a significant correlation between GFAP levels in the CSF and age in years. (B) no significant correlation with CADES scores. (C) no significant correlation with CCDR scores. (D) no significant correlation with GFAP levels in plasma. (E) through (G) are GFAP values in the plasma plotted against age (E), CADES scores (F), and CCDR scores (G). No significant correlation.

We next examined the $A\beta_{1-42} / A\beta_{1-40}$ ratio. In both the CSF and plasma. The plasma ratio was significantly negatively correlated with age ($p = 0.025$, $r = -0.47$, Figure 5). This finding reflects a worsening $A\beta$ ratio with aging, consistent with increasing amyloid pathology. The CSF $A\beta_{1-42} / A\beta_{1-40}$ ratio was also significantly negatively correlated with CADES scores ($p = 0.011$, $r = -0.52$), indicating that lower ratios in the CSF are associated with more severe cognitive impairment. Interestingly, this correlation was not observed using plasma measurements, suggesting that while plasma amyloid measurements reflect systemic amyloid dynamics, the CSF ratio may serve as a more sensitive marker for cognitive decline in CCD.

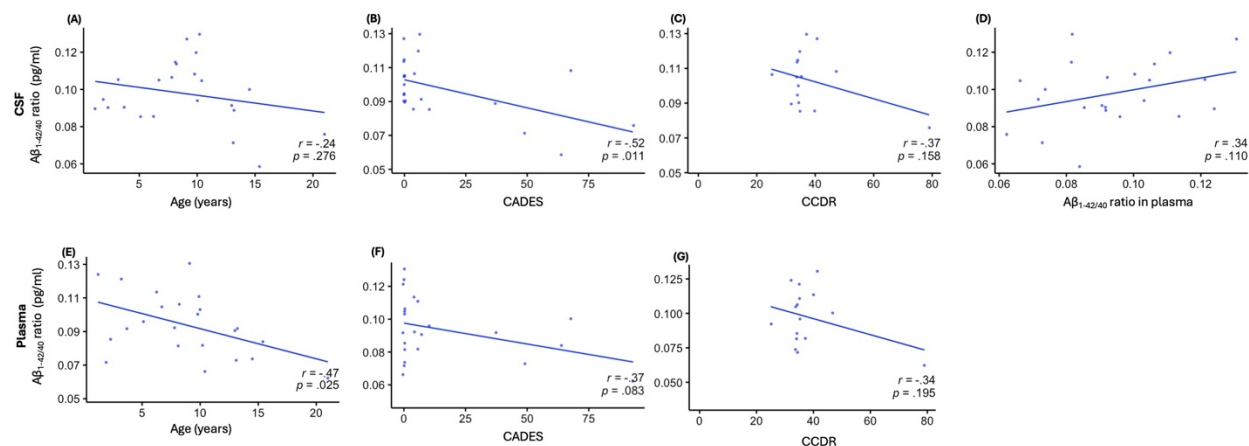


Figure 5. Correlation of Beta-Amyloid ($A\beta_{42/40}$) Ratios in CSF & Plasma vs. Age, CADES, and CCDR. Scatter plots illustrating the relationship between Beta-Amyloid ($A\beta_{1-42} / A\beta_{1-40}$) ratios in cerebrospinal fluid (CSF) and plasma and the clinical metrics age, CADES, CCDR, and in aged dogs. This figure assesses the potential of $A\beta_{1-42} / A\beta_{1-40}$ as a biomarker for canine cognitive dysfunction (CCD). (r = Pearson's coefficient, P = statistical significance value). (A) through (D) are $A\beta_{1-42} / A\beta_{1-40}$ ratio values in the CSF plotted against age, CADES scores, CCDR scores, and $A\beta_{1-42} / A\beta_{1-40}$ ratio values in the

plasma (D). (A) significant negative correlation between $A\beta_{1-42} / A\beta_{1-40}$ ratio values in the CSF and age in years. (B) significant negative correlation with CADES scores. (C) no significant correlation with CDDR scores. (D) no significant correlation with $A\beta_{1-42} / A\beta_{1-40}$ ratio values in plasma.

NfL concentrations in CSF and plasma were significantly positively correlated with age ($p = <0.001$, <0.001 ; $r = 0.71$, 0.73 , respectively), reinforcing NfL's potential as a biomarker for aging and cognitive decline in dogs. Notably, CSF and plasma NfL concentrations showed a strong positive correlation with CADES scores ($p = <0.001$, <0.001 ; $r = 0.48$, 0.76 , respectively, Figure 6), suggesting that higher peripheral NfL concentrations are associated with greater cognitive impairment in both the CSF and plasma. Importantly, we observed a significant positive correlation between NfL concentrations in plasma and CSF ($p = 0.016$, $r = 0.47$, Figure 6), suggesting that plasma NfL levels can guide central nervous system pathology. Expanding on this finding, we ran a multiple regression analysis to determine the role of age in influencing the association between NfL values in the CSF and the plasma. After including age in the model, the association between plasma and CSF NfL was positive but did not reach statistical significance (Table 2). Additional Correlation analyses between individual biomarkers are listed in Figure 7.

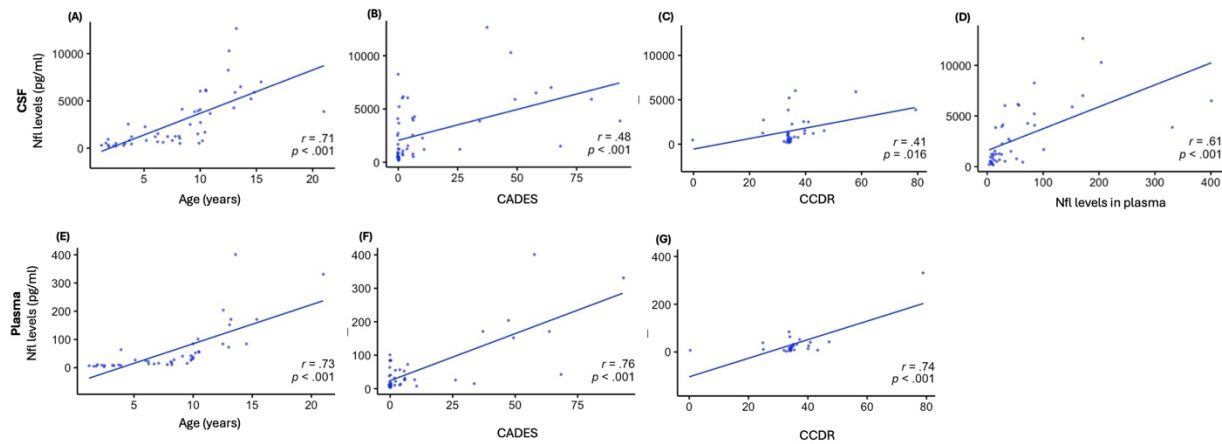


Figure 6. Correlation of NFL Levels in CSF and plasma vs. Age, CADES, and CCDR. Scatter plots depicting the levels of Neurofilament Light Chain (NFL) in cerebrospinal fluid (CSF) plotted against age, CADES, CCDR, weight, GFAP levels, and A β 42/40 ratios in dogs. These plots explore the relationship between NFL concentrations and other clinical and biochemical markers in CCD. (r = Pearson's coefficient, P = statistical significance value). (A) through (D) are NFL values in the CSF plotted against age, CADES scores, CCDR scores, and NFL values in the plasma. (A) significant positive correlation between NFL values in the CSF and age in years. (B) no significant correlation with CADES scores. (C) no significant correlation with CCDR scores. (D) significant positive correlation with NFL values in plasma. (E) through (G) are NFL values in the plasma plotted against age (E), CADES scores (F), and CCDR scores (G). (E) Significant positive correlation between NFL values in plasma and age in years. (F) significant positive correlation with CADES. (G) no significant correlation with CCDR.

(A)

Model	R-Squared	Adjusted R-Squared	F-value (2, 41 DF)	SD of the estimate	p-value (F-statistic)
Values	0.6567	0.6399	39.21	0.7047	<0.001***

(B)

Coefficients	Estimate	Standard error	t-value	p-value
Intercept	5.26799	0.35004	15.050	<0.001***
Age	0.15186	0.05101	2.977	0.004**
log(NfL in Plasma)	0.25678	0.18863	1.361	0.18087

Table 2. Summary of the linear regression analysis predicting log-transformed cerebrospinal fluid NFL (log(NfL in CSF)) from Age and log-transformed plasma NFL (log(NfL Plasma)). The model was specified as: $\log(\text{NfL in CSF}) = \beta_0 + \beta_1 \cdot \text{Age} + \beta_2 \cdot \log(\text{NfL in Plasma})$. The results indicate that Age is a statistically significant predictor of log(NFL_CSF), while log(NfL in Plasma) does not contribute significantly. This summary highlights the key parameter estimates, their associated standard errors, t-values, and p-values.

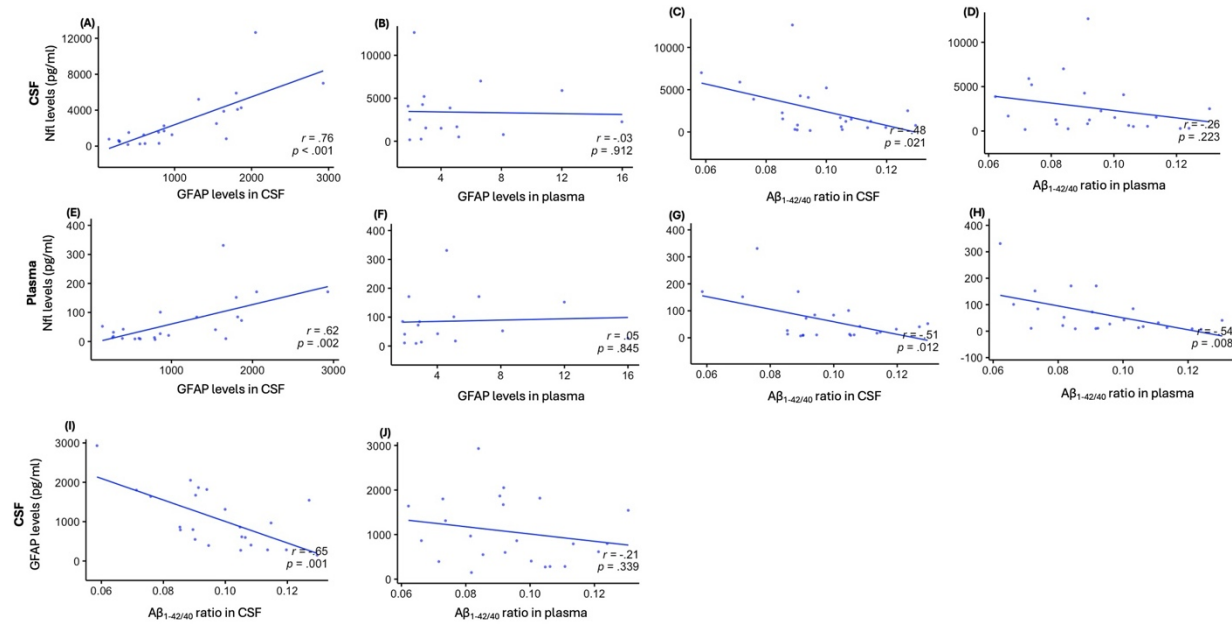


Figure 7. Correlation analyses among GFAP, Beta-Amyloid ($A\beta_{42/40}$) Ratios, and NfL levels in CSF and plasma. Scatter plots illustrate pairwise correlations between glial fibrillary acidic protein (GFAP), neurofilament light chain (NfL), and amyloid- $\beta_{1-42/40}$ ($A\beta_{1-42/40}$) ratio in cerebrospinal fluid (CSF) and plasma. (A–B) NfL levels in CSF are plotted against GFAP levels in CSF (A) and plasma (B). (C–D) NfL levels in CSF are plotted against $A\beta_{1-42/40}$ ratio in CSF (C) and plasma (D). (E–F) NfL levels in plasma are plotted against GFAP levels in CSF (E) and plasma (F). (G–H) NfL levels in plasma are plotted against $A\beta_{1-42/40}$ ratio in CSF (G) and plasma (H). (I–J) GFAP levels in CSF are plotted against $A\beta_{1-42/40}$ ratio in CSF (I) and plasma (J). Pearson's correlation coefficient (r) and p -values are shown in each panel. Significant correlations ($p < 0.05$) are observed in panels A, C, E, G, H, and I.

2.5 Discussion

Aging is the most significant risk factor for neurodegenerative diseases in humans, such as AD (Land et al., 2021). While traditional laboratory models often fail to fully capture the complexity of natural aging, dogs provide an excellent translational model for studying cognitive decline and neurodegeneration (Head, 2013). CCD typically manifests in dogs over the age of 10 and shares many pathological features with AD, including amyloid angiopathy and increased amyloid burden (Smolek et al., 2016). Furthermore, the severity of CCD increases with age, much like AD in humans. The median lifespan of beagles, a commonly studied breed, is approximately 13.9 years (Greer et al., 2007), and canine age ranges correspond to human aging stages: < 5 years in dogs (~< 40 years in humans), 5-9 years (~40-60 years), and > 9 years in dogs (~60 years in humans) (Giacomucci et al., 2022). The compressed lifespan makes dogs better suited to study brain aging and related neurodegenerative processes.

The current diagnosis of CCD is primarily based on owner-directed surveys and the exclusion of other neurological or systemic conditions. Thus, developing a diagnostic test that can be performed through a simple blood draw during routine veterinary visits would be highly beneficial for both owners and dogs. Based on prior research in both dogs and humans, we hypothesized that biomarkers such as NfL, GFAP, and A β isoforms could be correlated with behavioral and pathological markers of cognitive decline in aging dogs.

Biochemical assays demonstrated age related increase of A β ₁₋₄₂ in the frontal cortex in senior dogs (Figure 1). These findings align with previous studies on dogs with

CCD (Head et al., 2010; Urfer et al., 2021), which also observed increased amyloid pathology using similar biochemical techniques. Notably, no significant changes were detected in tau phosphorylation at the S396 site, which could be attributed to the sensitivity of the assay used in dogs or the specific brain region analyzed.

Among the biomarkers evaluated, plasma NfL emerged as a promising candidate. It was significantly associated with age, cognitive impairment (as measured by CADES and CCDR scores) and CSF NfL levels. The strong association between plasma and CSF NfL levels suggests that peripheral measurements may reflect central neurodegenerative processes. However, after adjusting for age in a multiple regression model, this association lost statistical significance, indicating that age may confound this association. These findings align with established data from human studies of AD and other dementias, where biomarkers like NfL have been shown to be an early indicator of cognitive decline, mild cognitive impairment (Giuffrè et al., 2023), AD (Legdeur et al., 2018), and frontotemporal dementia (Tapp et al., 2004). Aging is a strong prognostic factor for cognitive impairment in both humans (Yun et al., 2021) and dogs (Fowler et al., 2024). In this study, we showed that age was significantly associated with several dementia biomarkers, NfL, GFAP and A $\beta_{1-42/1-40}$ ratio.

Although NfL is a promising biomarker for cognitive decline and neurodegeneration in humans, it is a non-specific neuronal protein that can be elevated due to various brain injuries or diseases. Increased plasma NfL levels have been documented in canine meningoencephalitis (Yun et al., 2023), epilepsy (Perino et al., 2021), hypoxic injury (Chatterjee et al., 2022), and even normal aging in dogs (Shir et al., 2022). Therefore, it is essential to pair NfL changes with other biomarkers and

clinical assessments for accurate CCD diagnosis. In this context, we assessed the $A\beta_{1-42}/A\beta_{1-40}$ ratio and GFAP levels. $A\beta$ ratio, known to decrease in human patients with AD and other dementias, decreased in both plasma and CSF of aged dogs with CCD. However, it was not statistically significant in plasma (Figure 2). In addition, GFAP, a protein abundant in astrocytes, has been identified in plasma from human patients with early and late-stage AD (Cicognola et al., 2021). In our study, we observed a slight but not significant increase in GFAP levels in CSF and plasma samples from CCD+ dogs (Figure 2), further highlighting the role of glial activation in CCD pathology.

It is important to note that our study is based on association analyses rather than mechanistic investigations. The findings indicate that a panel of biomarkers, rather than a single marker, are necessary to accurately reflect the complex neurodegenerative processes underlying CCD pathology. Future studies with larger sample sizes are warranted to validate these biomarkers and to further elucidate the biological changes in CCD.

Overall, these results prove the translational value of the canine model for investigating neurodegeneration. Through establishing associations between peripheral and central biomarkers and cognitive decline, our study lays the groundwork for the development of minimally invasive diagnostic tools that may eventually inform both veterinary practice and human AD research.

2.6 Conclusion

Diagnosing CCD is crucial for several reasons, including effective management for dog owners and clinicians. Importantly, the proper diagnosis of CCD will allow researchers to understand disease progression related to cognitive decline and behavioral changes and develop therapeutics to intervene earlier in disease, increasing the health span of these animals. Also, due to dogs aging naturally and spontaneously acquiring neurodegenerative diseases similar to AD and AD related dementias, a set of diagnostic tools, including NfL, GFAP, $A\beta_{1-42}/A\beta_{1-40}$ biomarkers paired with clinical, behavioral, and pathological changes will allow researchers to better understand the progression of disease in a model with a shortened lifespan, compared with humans.

Our findings demonstrate that biomarkers such as NfL, GFAP, and $A\beta_{1-42}/A\beta_{1-40}$ ratio correlate with pathological changes and cognitive impairment in aging dogs, aligning with similar observations in human neurodegenerative diseases like AD. While NfL shows promise as a diagnostic tool, its non-specificity underscores the importance of combining it with additional biomarkers and clinical assessment tools for accurate CCD diagnosis.

These results provide critical insights into the pathophysiology of CCD, advancing our understanding of disease mechanisms and offering a framework for developing early diagnostic tools and therapeutic interventions. Leveraging biomarkers in naturally aging canine models not only benefits canine health but also enhances translational research, bridging the gap between preclinical studies and human clinical applications. By refining diagnostic and therapeutic approaches, we can improve the

health of aging dogs and gain valuable insights applicable to human aging and neurodegenerative diseases.

Chapter Three: Alzheimer's Disease Neuropathological characterization of the dog brain and relationship to biofluid biomarkers and cognitive function

Abdullatif Alsulami^{1,2}, Marika Bogdani³, Evan MacLean⁴, C. Dirk Keene³, Stephanie McGrath^{5,2}, Dog Aging Project Consortium, Caitlin S. Latimer^{3*} and Julie A. Moreno^{1,2*}

¹Department of Environmental & Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

²Brain Research Center, Colorado State University, Fort Collins, CO, USA

³Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, USA

⁴Department of Psychology, College of Veterinary Medicine, University of Arizona, Tucson, USA

⁵Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

3.1 Abstract

Alzheimer's disease (AD) is a progressive neurodegenerative disorder and a major global health challenge affecting more than 55 million people worldwide. AD is clinically defined by progressive cognitive decline and neuropathologically characterized by the accumulation of amyloid-beta ($A\beta$) plaques and neurofibrillary tangles (NFTs), yet effective disease modifying therapies remain elusive. While transgenic rodent models have provided valuable mechanistic insights, they do not fully capture the spontaneous, age-related nature of human AD. In contrast, the companion dog develops naturally occurring age-associated cognitive decline and AD-like neuropathological features, including $A\beta$ deposition and tau pathology, and behavioral impairments measurable by the validated cognitive scales. However, the systematic application of human AD neuropathological criteria to canine brains has been limited. The objective of this study was to apply established human neuropathological criteria (Thal, Braak, and CERAD) to aged canine brains and examine relationships among neuropathology, cognitive status, and plasma biomarkers. Postmortem brain tissues from 24 client-owned senior dogs were evaluated using Thal phases for $A\beta$ deposition, Braak-based regional assessment of tau pathology, and CERAD criteria for neuritic plaques. Neuropathological findings were integrated with antemortem owner-reported cognitive assessments and plasma biomarker measurements to evaluate the clinico-pathologic and biomarker associations. Senior dogs exhibited $A\beta$ deposition consistent with early to intermediate Thal phases, variable and regionally restricted tau pathology, and an absence of neuritic plaques. Quantitative analysis demonstrated greater $A\beta$ burden in cognitively impaired dogs compared with cognitively intact dogs, while age, but not cognitive score, was

significantly associated with regional A β burden. Further, plasma glial fibrillary acidic protein (GFAP) levels showed a significant positive correlation with A β plaque burden, whereas the other plasma biomarkers assessed did not. Senior-aged dogs exhibit neuropathologic features consistent with early-stage AD-like pathology, characterized by A β deposition and limited tau pathology in the absence of neuritic plaques. These findings support the utility of the companion dog as a naturally aging model for investigating early AD-related pathological processes and evaluating translational biomarkers during preclinical disease.

3.2 Background

Alzheimer's disease (AD), a progressive neurodegenerative disorder, affects over 55 million individuals worldwide and is projected to triple by 2050, increasing social and economic burdens (Shin, 2022). AD is characterized by progressive cognitive decline and neuropathologic features, collectively termed Alzheimer's disease neuropathologic change (ADNC), include amyloid- β (A β) plaques and tau neurofibrillary tangles (NFTs). AD lacks effective disease-modifying therapies, underscoring the urgent need for innovative research approaches (Lei et al., 2021). Progress in the field has been hindered by limitations in transgenic rodent models, which, despite providing mechanistic insights, often rely on genetic modifications and fail to fully recapitulate the spontaneous, age-related, and multifactorial nature of human AD pathology (Drummond & Wisniewski, 2017). These limitations highlight the need for alternative animal models that better capture the complex interplay of genetic, environmental, and aging factors in AD.

The companion dog represents a distinctive and naturally occurring model of AD, exhibiting age-dependent cognitive decline and neuropathological features that overlap with key elements of human ADNC, including age associated brain atrophy and the accumulation of A β plaques and NFTs (Abey et al., 2021a; De Sousa et al., 2023; Dewey et al., 2021; Schütt et al., 2016a). In contrast to traditional rodent models, dogs share human environments, lifestyles, and daily exposures, providing a naturalistic and translationally relevant context for studying the development and progression of AD-like pathology, but on a restricted timescale. On average, the domestic dog lives

approximately 10–12 years compared to an average human lifespan of 77 years in some populations (Galvani-Townsend et al., 2022; Montoya et al., 2023).

Importantly, dogs experience age-associated cognitive decline, termed canine cognitive dysfunction (CCD) syndrome, which typically affects dogs over eight years old and is associated with signs of dementia that often occur in AD, including disorientation, altered social interactions, and functional decline (Prpar Mihevc & Majdič, 2019b). Recent studies from the Dog Aging Project (dogagingproject.org) have advanced our understanding of CCD epidemiology by assessing signs of dementia in over fifteen-thousand dogs, revealing strong associations between CCD and factors such as age (Yarborough et al., 2022) and physical activity (Bray et al., 2023). Pathologically, A β accumulates progressively with age in dogs, both as extracellular plaques and soluble oligomers, mirroring AD-like pathology with changes in amyloid precursor protein (APP) processing that favors amyloidogenic cleavage (Pop et al., 2012). Tau pathology has generally been shown to be limited in canines, however, recent studies leveraging improved phospho-tau–specific reagents demonstrate regionally restricted tau pathology, particularly within limbic structures (Abey et al., 2021a). Together, these observations suggest that dogs develop both clinical and neuropathological features relevant to AD. Despite this convergence, a critical gap remains: rigorous studies directly linking age-associated cognitive decline in dogs to the extent and regional distribution of AD-like neuropathology are lacking. This disconnect is due in part to the absence of a systematic, standardized framework for staging canine A β and tau pathology that is comparable to those established for human AD.

To assess clinical behavioral changes in dogs, validated behavioral assessment tools are used, such as the Canine Dementia Scale (CADES) and Cognitive Cognitive Dysfunction Rating (CCDR) scale. These scales measure behavioral impairments in aged dogs and have similarities to human AD scoring systems like the Clinical Dementia Rating (CDR) (Bray et al., 2023; Madari et al., 2015; Olby et al., 2025; Salvin et al., 2011). Through evaluation of domains such as disorientation, social interaction, and daily functioning, these tools have revealed progressive cognitive decline in aging dogs, positioning them as a translational bridge between rodent models and human clinical studies (Chapagain et al., 2018; Haake et al., 2024). However, without corresponding standardized neuropathologic staging, the relationship between these clinical measures and underlying brain pathology remains poorly defined.

Although behavioral changes in aging dogs have been well described, comprehensive neuropathological mapping aligned with human AD frameworks (Montine et al., 2012) is lacking, hindering full validation of the canine model for AD. To address this gap, we applied established human AD neuropathological criteria, Thal phases for A β deposition, Braak staging for NFT distribution, and Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria for neuritic plaques assessment, to characterize AD-like pathology using client owned dogs (Montine et al., 2012). We further integrated plasma biomarkers used in human AD diagnosis that are also altered in CCD to examine their relationships with neuropathology and cognitive function (Alsulami et al., 2025a; Fefer et al., 2022; Panek et al., 2020). By systematically aligning canine neuropathology with human AD staging and linking these findings to antemortem cognitive and biomarker assessments, this study aims to establish a unified

clinicopathologic framework for canine AD-like disease. Our findings demonstrate that AD-like pathology in aged dogs follows patterns similar to those observed in humans and that this pathology is associated with cognitive status. Through the application of standardized neuropathologic assessment, this work provides a structural foundation for validating the companion dog as a translational model of AD.

3.3 Methodology

Recruitment and clinical assessment

Dogs were selectively recruited through multiple channels: social media and local news platforms, referrals from a network of family veterinarians identifying cases of suspected Canine Cognitive Dysfunction (CCD), and from the existing population of senior dogs evaluated by the Neurology Clinical Trials Service at the Colorado State University Veterinary Teaching Hospital, and from the Dog Aging Project's Brain Health Study (McGrath et al., 2025). Informed consent was obtained from all pet owners prior to study enrollment. The inclusion criteria required absence of brain tumors or other neurological conditions that could affect cognition. Clinical assessments included the Cognitive Cognitive Dysfunction Rating (CCDR) Scale (Salvin et al., 2011), a validated survey instrument that measures signs of CCD. Details regarding age, and cognitive scores of participating dogs are listed in Table 1. In this study, we used a moderately relaxed criterion cutoff for dogs with a CCDR score >40 were classified as CCD-positive (CCD+), and those with a score ≤ 40 were classified as CCD-negative (CCD-).

Collection of plasma

Whole blood was collected, placed into EDTA blood collection tubes and centrifuged to separate plasma. Samples were stored at -80C until used for experimentation. Plasma biomarker measurements were available for a subset of the full neuropathology cohort due to sample availability.

Biofluid Biomarker Assay

The Quanterix SR-X platform was used to simultaneously measure Abeta 40 (A β 40), Abeta 42 (A β 42), Glial Fibrillary Acidic Protein (GFAP), and Neurofilament light chain (NF-L) in antemortem canine plasma samples (Simoa® Neurology 4-Plex E Advantage Kit). Plasma samples were thawed, centrifuged, and diluted 4X in plasma diluent provided by the kit. Standards (in triplicate), and diluted samples (in duplicate) were loaded onto the plate and the assay performed according to the Simoa® Neuro 4-Plex Advantage Plus kit for SRX instructions. Briefly, antibody conjugated beads and secondary detection antibodies were added to the plate and incubated overnight. After plate washing, activated RGP reagent was added, and the plate was incubated for 10 minutes. After washes and open air drying the plate was loaded onto the SRX. Quantification was performed using Quanterix SRX software version 1.2.0 using a 5-parameter sigmoidal curve generated from the standards. All sample aliquots were thawed only on the day of the assay, used once and discarded. Samples with responses above the dynamic range of the calibration curve were diluted 10X, rerun and reanalyzed.

Tissue collection and processing

Twenty-four canine brains were obtained postmortem after euthanasia and necropsy from privately owned dogs with owner consent. The brains were collected at the Veterinary Teaching Hospital at Colorado State University or the University of Washington and fixed in 10% neutral buffer formalin for at least 72 hours. Tissue blocks containing pre-frontal cortex, visual cortex, striatum, hippocampus, midbrain and cerebellum were processed using a Leica TP1020 Automatic Benchtop Tissue Processor, embedded in paraffin wax (Cancer Diagnostics, Cat #: EEPAR56), and sectioned on a Thermo Scientific HM 325-2 Manual Microtome at 5 μ m thickness and mounted on positively charged glass slides (Superfrost Plus, Cancer 232 Diagnostics, Cat #: 4951) for staining and analysis.

Immunohistochemistry

Immunohistochemical staining was performed to evaluate the pathologic hallmarks of ADNC. A β plaques were detected using Anti- β -Amyloid (1-16, 1:5000) on a Leica BondMax automated stainer with citrate antigen retrieval (PH 6.0). NFTs were evaluated using phospho-Tau (Thr181) Monoclonal Antibody (Thermo Fisher MN1050, 1:200) with manual staining. Briefly, paraffin-embedded sections were dewaxed in xylene and rehydrated through graded ethanol before antigen retrieval in 0.01M sodium citrate for 20 minutes at 95°C. Endoperoxides were removed by incubating sections in a 0.30% hydrogen peroxide and water solution for 30 minutes. To block non-specific labeling, sections were treated with 10% horse serum in Tris-A with 2% BSA (bovine serum albumin) and 2% Triton-X in Tris buffered saline (TBS). Sections were then incubated overnight at 4°C with primary antibodies diluted in Tris-A with 2% BSA. After

washing with Tris-A with 2% BSA, sections were incubated for one hour with their corresponding biotinylated secondary antibodies (goat anti-rabbit or horse anti-mouse, Vector Labs) diluted in 10% horse serum in Tris-A with 2% BSA at 1:250. Slides were counterstained with hematoxylin, rinsed in bluing reagent, dehydrated through graded ethanol followed by xylene, and mounted with mounting media. Coverslips were applied using 1 cover glass.

To further evaluate classical AD neuropathological features, modified Bielschowsky silver stain was performed manually (Yamamoto & Hirano, 1986). Briefly, formalin fixed paraffin-embedded blocks were dewaxed in xylene, rehydrated through graded ethanols and incubated in fresh 20% silver nitrate for 20 minutes. Sections were then incubated in titrated ammoniacal silver solution and developed in a formalin-based developer. The reaction was monitored microscopically to optimize contrast. Slides were rinsed in distilled water extensively, treated with 2% sodium thiosulfate to remove unreduced silver, dehydrated through graded ethanols followed by xylene, and mounted with mounting media.

Histology Assessment

Neuropathological evaluation was performed by adapting National Institute on Aging-Alzheimer's Association guidelines for AD neuropathology (Montine et al., 2012) to aging canines. All assessments were conducted blinded to clinical data, including animal age and cognitive scores. Histological assessment of A β deposits, NFTs, and neuritic plaques density was carried out on all dogs included in the study. An "ABC" score for neuropathological changes in canine brains was scored based on: (A) A β

deposits, (B) NFTs, and (C) neuritic plaques. Brain sections analyzed for each assessment are detailed in Supplemental Figure 1.

Amyloid- β Pathology Assessment

We evaluated six regions based on Thal's 2002 staging criteria (Thal et al., 2002) : Middle frontal gyrus and primary visual cortex (Thal Phase 1), hippocampus (Thal Phase 2), neostriatum (Thal Phase 3), midbrain (Thal Phase 4), and cerebellum (Thal Phase 5). All assessments were conducted blinded to cognitive scores. For each region, we ensured that the tissue slices sampled comparable anatomical areas across all dogs. A brain region was considered positive for amyloid pathology if one or more plaques were identified in the tissue examined.

Semi-quantitative amyloid burden: Pathology assessment was performed using four categories:

0 = no pathology,

1 = pathology covers less than half the tissue,

2 = pathology covers approximately half the tissue,

3 = pathology covers the entire tissue.

Phosphorylated Tau Pathology Assessment

We assessed tau pathology for NFTs based on Braak staging criteria (Braak & Braak, 1991) in trans-entorhinal cortex (Braak stage I), CA1 (Braak stage II), subiculum (Braak stage III), entorhinal cortex (Braak stage IV), frontal cortex (Braak stage V), and primary visual cortex (Braak stage VI). An area was considered positive for pathology if morphological NFTs were identified using an immunohistochemical stain Thr181.

Neuritic Plaque Density Assessment

We assessed neuritic plaque density using the Consortium to Establish a Registry for Alzheimer's Disease (CERAD) criteria (Mirra et al., 1991) by evaluating the prefrontal cortex with the Bielschowsky silver stain. A neuritic plaque is characterized by the presence of dystrophic neurites that are typically highlighted on silver stain. The density is assessed as the number of plaques per 100x field.

Digital Image Analysis

To quantify the burden of amyloid and phosphorylated tau (p-tau) pathology in the canine brain, histologic slides were digitized using an Aperio AT2 whole-slide scanner (Leica Biosystems; software version 102.0.7.5). All slides were scanned at 20× magnification using identical scanner settings (gain, brightness, and exposure) to minimize image-to-image variability. Regions of interest (ROIs) were manually delineated using HALO image analysis software (v3.4.2986; Indica Labs) and included hippocampal subfields and cortical gray matter.

For amyloid pathology, an area-based quantification approach was applied using the HALO Area Quantification module to calculate the percentage area occupied by positive staining within each ROI. Thresholding and masking parameters were optimized to accurately identify amyloid plaques while excluding nonspecific background staining and artifacts. For tau pathology, quantitative analysis was performed using Qupath software (version 0.6.0) (Bankhead et al., 2017a; Nüesch et al., 2025). Briefly, a machine learning object classifier was trained to identify p-tau181 positive cells. Cell counts were normalized to area (cells/mm²).

Statistical Analysis

Prior to conducting group comparisons, normality was assessed using the Shapiro-Wilk test, and Levene's test was performed to check for homogeneity of variance. For group comparisons, two-sample t-tests were used where assumptions of normality and homogeneity were met and Mann-Whitney test when normality assumption was violated. Spearman's correlation was computed to assess the association between CDR scores, dog age in years, and A β and biomarker concentrations. All statistical analyses were performed using R software (version 4.3.1) and GraphPad Prism (version 10.1.2), with statistical significance set at $p < 0.05$ for all analyses.

3.4 Results

The extent of neuropathological changes in our cohort of aged dogs (eight years or older) was evaluated using criteria adapted from the NIA–AA guidelines for the neuropathological assessment of AD, analyses described in Figure 1 (Montine et al., 2012). Specifically, neuropathological evaluation was performed using Thal's phases for A β deposition (Figure 2, panels A-F). All cases (100%) exhibited A β deposition in the neocortex. A β pathology was also observed in the hippocampus in 87.5% of cases, the striatum in 37.5%, and the midbrain in 8% of cases, while no A β deposits were detected in the cerebellum (Figure 3B).

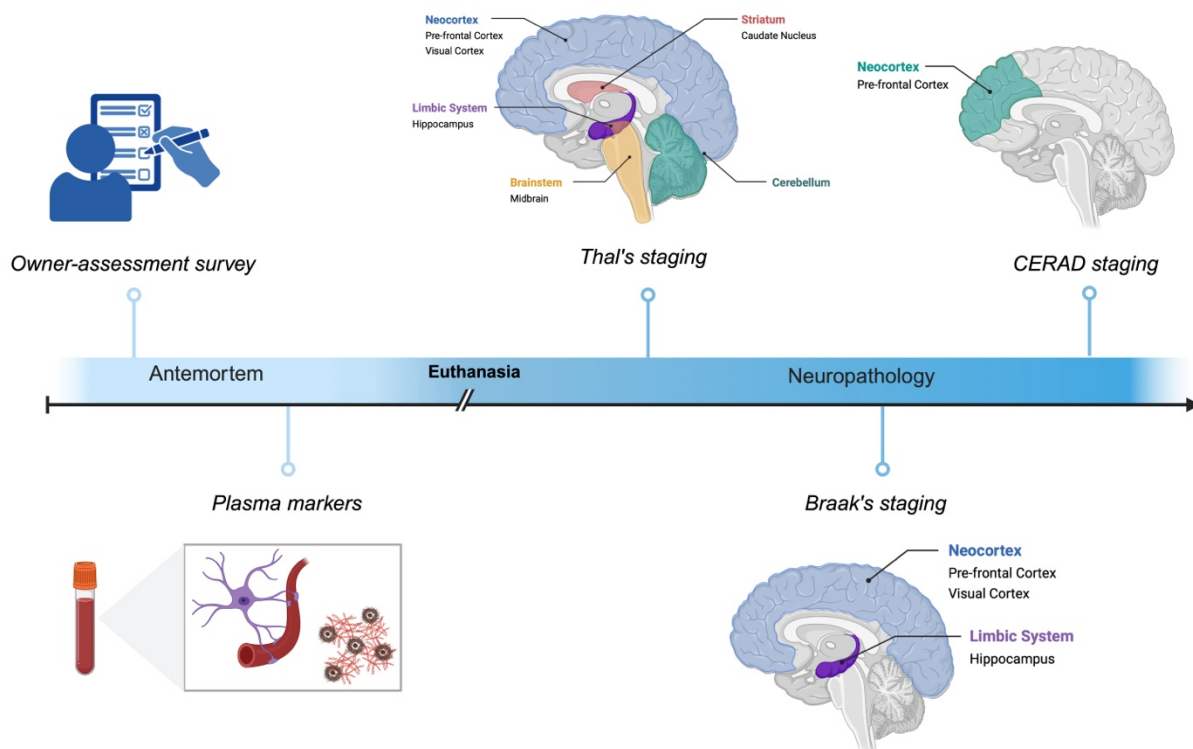


Figure 1. Study design and methodological overview. Schematic representation of the study workflow. Antemortem assessments included owner-based cognitive surveys and collection of plasma biofluid markers. Following euthanasia, postmortem neuropathological assessment was performed. Pathological evaluation included Thal's amyloid phase, Braak's neurofibrillary tangle stage, and CERAD scoring across multiple brain regions. Figure created with BioRender.

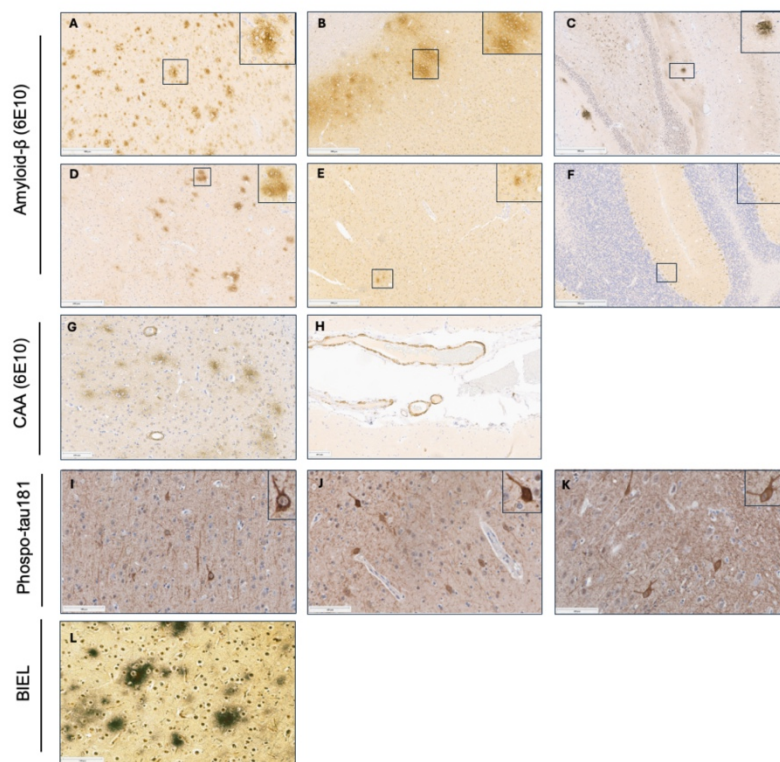


Figure 2. Senior canines show neuropathological characteristics similar to humans. Representative immunohistochemistry images showing Alzheimer's disease-like pathology in canine brains. Panels (A–F) show β -amyloid plaques (6E10 antibody) in the prefrontal cortex (A), visual cortex (B), hippocampus (C), striatum (D), midbrain (E), and cerebellum (F). Panels G and H show cerebral amyloid angiopathy (CAA) in

parenchymal vessels (G) and leptomeninges (H). Panels (I–K) show neurofibrillary tangles (NFTs) visualized with phospho-tau (p-tau181) antibody in the prefrontal cortex (I), visual cortex (J), and hippocampus (K), with insets highlighting positive cells. Panel (L) shows Bielschowsky silver staining in prefrontal cortex. Scale bars = 500 μ m panels A–F, and 100 μ m panels G–L.

Neuropathologic examination of tau pathology was modeled after Braak regional staging principles using immunohistochemistry for phosphorylated tau (pTau181) (Figure 2 I–K). Tau pathology was less pervasive than amyloid, with 71% of animals exhibiting neurofibrillary tangle pathology. The hippocampus, particularly CA1, was most frequently involved (62.5%), with fewer animals showing extension into the frontal or primary visual cortices (46%) (Figure 3B). Among animals with cortical involvement, most exhibited a hippocampus-to-neocortex progression consistent with Braak staging; however, 17% demonstrated atypical patterns, including neocortical involvement without hippocampal tangles or non-contiguous regional involvement.

Using the CERAD criteria, we evaluated brains for neuritic plaques. Despite widespread A β deposition detected by immunohistochemistry, no plaques meeting the morphologic criteria for neuritic plaques were identified by Bielschowsky silver staining. Accordingly, all animals received a CERAD score of absent (Figure 2L).

To generate a comprehensive neuropathological score for each animal we combined the Thal, Braak and CERAD scores in accordance with the 2012 NIA-AA guidelines, adapted for canine neuropathology (Montine et al., 2012). Based on these criteria, the majority (80%) were classified as having LOW ADNC, while 16% met criteria for INTERMEDIATE ADNC; no animals met criteria for HIGH ADNC (Table 1).

One animal exhibited Thal phase 3 amyloid deposition with neurofibrillary tangles restricted to the primary visual cortex. Because this pattern does not align with Braak staging assumptions, this case was excluded from ADNC classification.

Cerebral amyloid angiopathy (CAA) was observed in 9 dogs. Vascular-associated A β deposition was most prominent in posterior regions, particularly the occipital (visual) cortex, with a smaller subset showing involvement of both visual and frontal cortices.

Regional differences in amyloid burden were first assessed using a semi-quantitative scoring approach. The prefrontal cortex exhibited the highest plaque burden, followed by the hippocampus and visual cortex, while the striatum and midbrain showed minimal pathology (Figure 3A). Across individual dogs, β -amyloid deposition was moderate to high in cortical and hippocampal regions, with considerable inter-individual variability (Figure 3B). Compared to amyloid pathology, tau pathology was less widespread and appeared more variable between cases (Figure 3B).

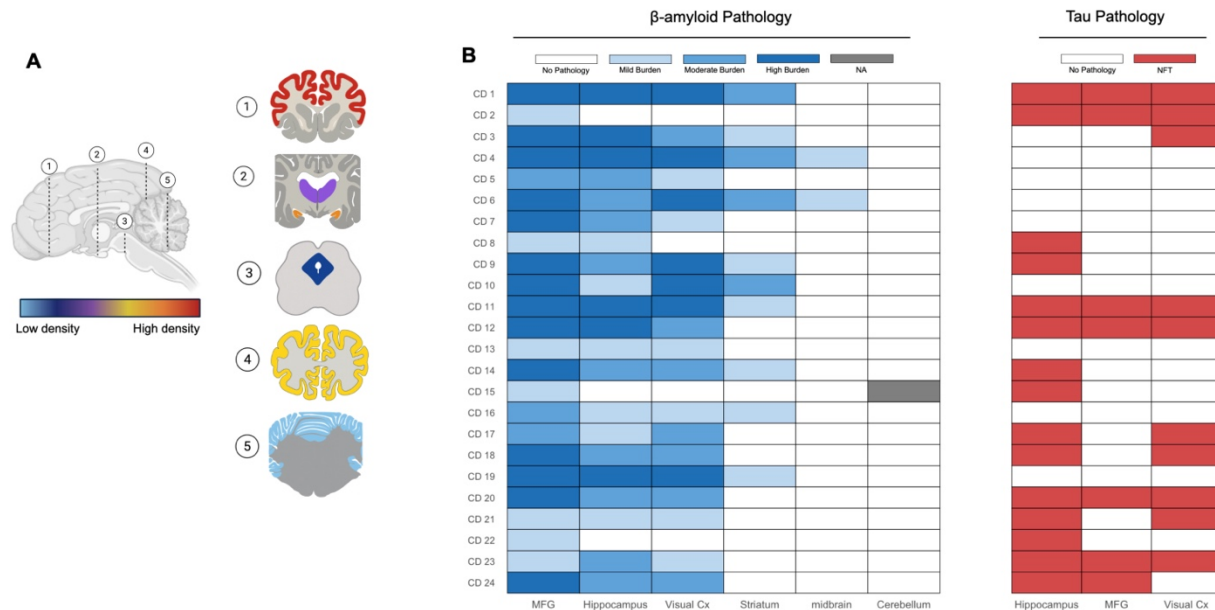


Figure 3. Regional distribution of β -amyloid and tau pathology in dogs. (A) Coronal brain sections illustrating the relative density of β -amyloid plaques, color-coded from absent pathology (light blue) to high pathology burden (red) based on semi-quantitative scoring. (B) Heatmap summarizing individual canine cases (CD1-24) for β -amyloid pathology (graded as no pathology, mild, moderate, or high burden) across neocortical and subcortical regions. Tau pathology is shown as the presence or absence of early neurofibrillary tangles (NFTs). Neocortical regions were predominantly affected by β -amyloid plaques, whereas hippocampus exhibited a higher burden of NFTs.

Quantitative analysis revealed a significantly higher $A\beta$ plaque burden in the prefrontal cortex of CCD+ dogs compared with CCD- dogs (Figure 4A). No statistically significant difference was observed in the visual cortex between groups (Figure 4C). In contrast, tau burden was significantly higher in the visual cortex of CCD+ dogs compared with CCD- dogs (Figure 4D), and no significant differences were detected in the prefrontal cortex (Figure 4B).

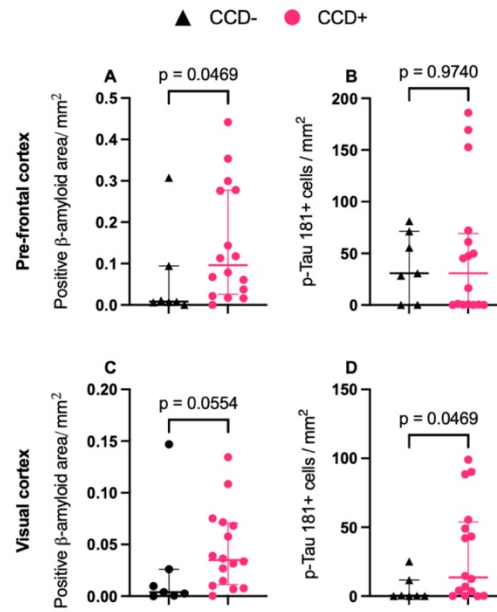


Figure 4. Region-specific increases in β -amyloid and P-tau181 pathology in cognitively impaired dogs. Quantitative assessment of β -amyloid pathology burden (A, C) and P-tau181 positive cells (B, D) in the pre-frontal and visual cortices of dogs with canine cognitive dysfunction (CCD+) and cognitively preserved dogs (CCD-). β -amyloid plaque burden was higher in the prefrontal cortex of CCD+ dogs (A; Hodges–Lehmann median difference 0.0582, 95% CI 0.00001 to 0.2592), whereas no difference was observed in the visual cortex (C; 0.0260, 95% CI -0.00018 to 0.0584). P-tau181–positive cells were increased in the visual cortex of CCD+ dogs (D; 12.42, 95% CI 0.0438 to 48.97), whereas no difference was observed in the pre-frontal cortex (B; -0.0051, 95% CI -31.45 to 49.61). Data are presented as median with interquartile range (IQR). Group comparisons were performed using Mann–Whitney tests.

Correlation analyses demonstrated a significant positive association between age and A β plaque burden in the visual cortex, but not in the prefrontal cortex. CCDR scores were not significantly associated with amyloid burden in either region (Figure 5). No significant association between tau pathology and age or CCDR scores were detected in either region (Figure 7).

To determine if the underlying neuropathologic changes in the aged dog brains could be predicted from antemortem plasma biomarkers we measured neurofilament light chain (NfL), A β_{1-40} , A β_{1-42} and glial fibrillary acidic protein (GFAP). These biomarkers have previously been shown by our group and others to be associated with age, cognitive status, and neurodegeneration in dogs (Alsulami et al., 2025a; Fefer et al., 2022; Panek et al., 2020). Plasma biomarker levels did not differ significantly between cognitively impaired (CCD+) and cognitively intact (CCD-) dogs for any of the analytes measured, including NfL, GFAP, A β_{1-40} , A β_{1-42} , or the A β_{1-42} /A β_{1-40} ratio (Figure 6).

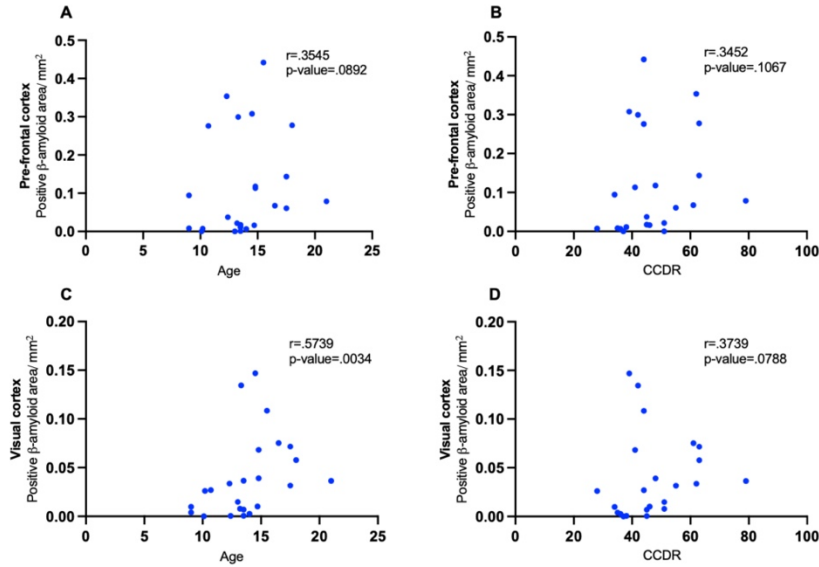


Figure 5. Association between β -amyloid plaque burden with age and cognitive scores.

Scatterplots showing relationships between β -amyloid plaque in the frontal cortex and age (A), and Canine Cognitive Dysfunction Rating (CCDR) scores (B), as well as β -amyloid plaque in the visual cortex and age (C) and CCDR scores (D). Spearman's rank correlation analysis showed no significant associations between CCDR scores and β -amyloid plaque burden in either brain regions. A positive moderate to strong association was observed between β -amyloid plaque burden in the visual cortex and age.

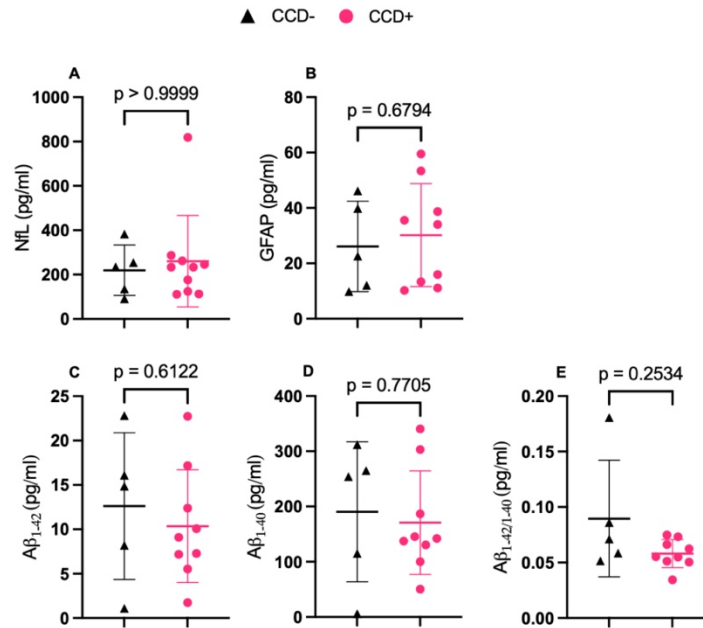


Figure 6. Plasma biomarker levels in aged dogs with and without cognitive dysfunction. Plasma concentrations of NfL (A), GFAP (B), A β ₁₋₄₂ (C), A β ₁₋₄₀ (D), and A β _{1-42/1-40} ratio (E) in dogs with canine cognitive dysfunction (CCD+) and cognitively preserved dogs (CCD-). No differences were observed between groups for any plasma biomarker (A; Hodges–Lehmann median difference 1.01, 95% CI -136.1 to 151.7; B; 1.29, 95% CI -23.86 to 26.63; C; -2.66, 95% CI -12.75 to 7.86; D; -14.77, 95% CI -166.34 to 131.57; E; -0.016, 95% CI -0.108 to 0.0039). No significant differences were detected. Data are presented as median with interquartile range (IQR). Group comparisons were performed using Mann–Whitney tests.

Despite the absence of group-level differences, correlation analyses revealed a significant positive association between plasma GFAP levels and regional A β plaque burden in both the prefrontal cortex and visual cortex (Figure 8C, D). In contrast, plasma NfL levels and the A β ₁₋₄₂/A β ₁₋₄₀ ratio did not show significant correlations with amyloid plaque burden in either brain region (Figure 8A, B, E, F). No significant associations

were detected between plasma biomarkers and tau pathology (Figure 9). These findings suggest that while plasma biomarker levels alone may not distinguish cognitive status in aged dogs, GFAP in particular may reflect underlying amyloid-associated neuropathology and serve as a potential marker of astroglial responses to A β deposition in this model.

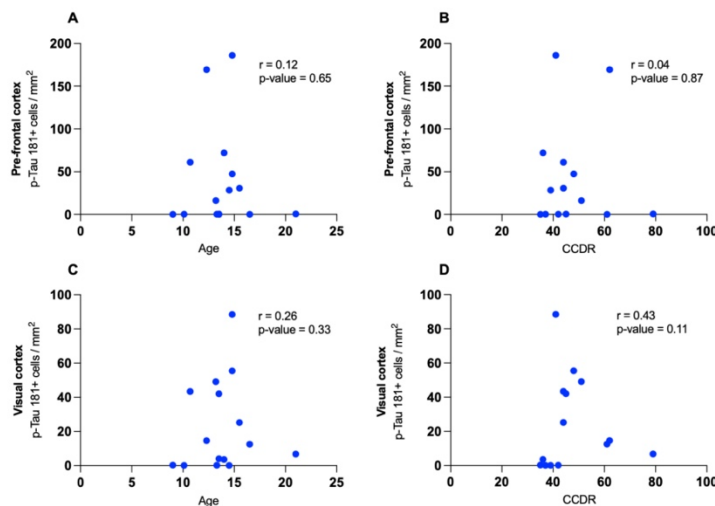


Figure 7. Association between P-tau181 positive cell counts with age and cognitive scores. Scatterplots showing relationships between P-tau181 counts in the frontal cortex and age (A), and Canine Cognitive Dysfunction Rating (CCDR) scores (B), as well as in the visual cortex and age (C) and CCDR scores (D). Spearman's rank correlation analysis showed no significant associations between CCDR scores and age with P-tau181 in either brain regions.

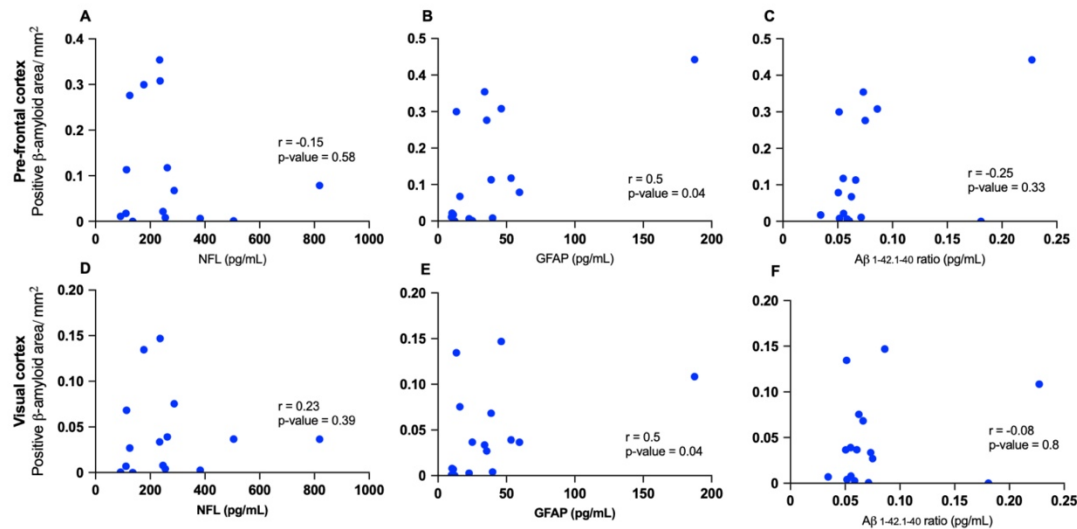


Figure 8. Association between plasma NfL, GFAP, and A β 1-42/1-40 ratio with β -amyloid plaque burden in aged dogs. Scatterplots show relationships between plasma neurofilament light chain (NfL) levels and β -amyloid plaque burden in the prefrontal cortex (A) and Visual cortex (B), plasma glial fibrillary acidic protein (GFAP) levels and β -amyloid plaque burden in the prefrontal cortex (C) and Visual cortex (D), and plasma A β 1-42/1-40 ratio and β -amyloid plaque burden in the prefrontal cortex (E) and Visual cortex (F). Spearman's rank correlation analysis showed a moderate association between GFAP levels and β -amyloid plaque burden in both regions.

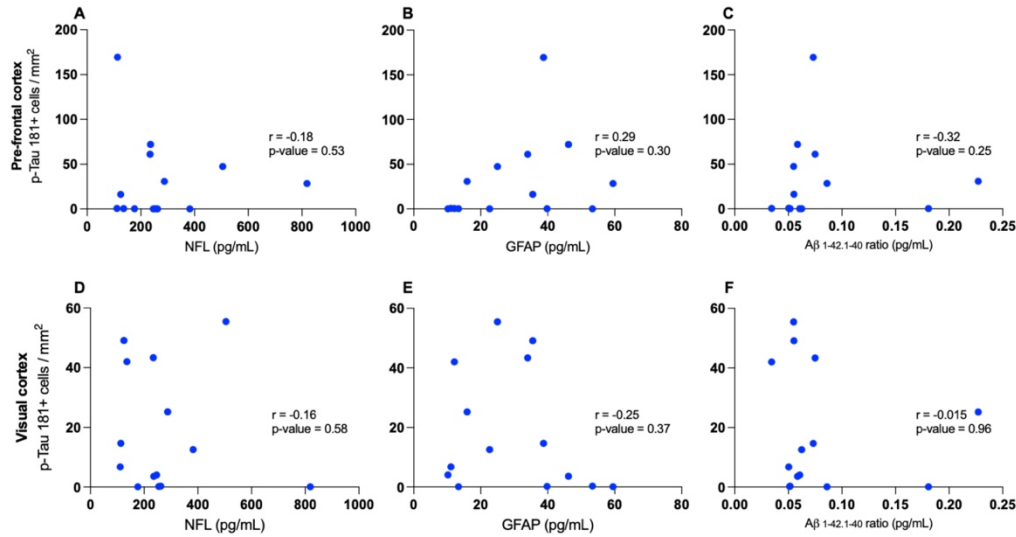


Figure 9. Association between plasma NfL, GFAP, and A β 1-42/1-40 ratio with P-tau181 positive cell counts in aged dogs. Scatterplots show relationships between plasma neurofilament light chain (NfL) levels P-tau181 positive counts in the prefrontal cortex (A) and Visual cortex (B), plasma glial fibrillary acidic protein (GFAP) levels and P-tau181 positive counts in the prefrontal cortex (C) and Visual cortex (D), and plasma A β 1-42/1-40 ratio and P-tau181 positive counts in the prefrontal cortex (E) and Visual cortex (F). Spearman's rank correlation analysis did not show significant associations between the biomarkers and P-tau181 positive counts in both regions.

3.5 Discussion

To determine whether and how the aging dog recapitulates key neuropathologic features of Alzheimer's disease (AD) we applied National Institute on Aging–Alzheimer's Association (NIA–AA) neuropathological criteria to dog brains. Using tools and approaches analogous to those used in human study, we found, consistent with prior reports, that dogs develop β -amyloid (A β) plaques in an age-dependent manner that closely parallels early amyloid pathology in humans as described in Figure 1. Notably,

the neocortex appeared to be the earliest site of plaque deposition, as all dogs in our cohort exhibited at least some cortical A β pathology. This pattern is consistent with findings from human autopsy studies, in which approximately 86% of individuals exhibit cortical amyloid deposition (Thal et al., 2002). Similar to humans, amyloid pathology in dogs followed a hierarchical regional distribution with involvement of the hippocampus after the neocortex, followed by the neostriatum, and, in rare cases, the midbrain. Importantly, amyloid deposition did not extend beyond Thal phase 2 in the majority of dogs, indicating that most aged dogs do not progress beyond the earliest stages of AD-like pathology (Figure 2). Consistent with this observation, no neuritic plaques, which represent a more mature and pathogenic form of amyloid deposition, were identified in any canine brain examined, further supporting the conclusion that aged dogs predominantly harbor early-stage amyloid pathology.

Neurofibrillary tangles (NFTs) were also identified in limbic and, less frequently, neocortical regions, indicating that tau pathology is present in canine cognitive dysfunction (CCD); however, tau pathology was less prevalent and less widespread than amyloid deposition and did not fully recapitulate the distribution observed in human AD. In this study, tau pathology was assessed using an antibody targeting phosphorylated tau at threonine 181 (pTau181) because the AT8 antibody, which is commonly used in human AD neuropathology, does not reliably label pathologic tau in the canine brain. Phosphorylation at Thr181 is considered an early tau modification in AD pathogenesis (Moloney et al., 2023). Together with the predominance of low Thal phases and the absence of neuritic plaques, these findings indicate that the dog brain

exhibits neuropathology resembling the very earliest stages of AD, positioning dogs as a unique model for investigating early disease mechanisms.

A major unresolved question in AD research concerns whether and how amyloid and tau pathology interact to drive disease progression. Our data provide insight into this relationship in a naturally aging model. All dogs with tau pathology also exhibited at least some degree of amyloid deposition, whereas not all dogs with amyloid pathology showed tau neurofibrillary tangles. Moreover, dogs with more severe amyloid pathology in the prefrontal cortex frequently exhibited greater tau burden in the hippocampus, and animals with hippocampal NFTs extending into neocortical regions typically had more advanced amyloid pathology (Thal phase ≥ 2) (Figure 3). This pattern supports the hypothesis that amyloid pathology facilitates, but is not sufficient for, the development and spread of tau pathology (van der Kant et al., 2020).

Quantitative analyses demonstrated that cognitively impaired dogs (CCD+) exhibited significantly greater A β plaque burden in the prefrontal cortex compared with cognitively intact (CCD-) dogs. In addition, CCD+ dogs showed significantly higher tau positive cells in the visual cortex, indicating that both A β and tau cortical pathologies are associated with cognitive impairment but may follow a region-specific pattern in the aging dog brain. However, some CCD- dogs displayed A β pathology consistent with Thal phase 2 and moderate to severe plaque burden. This may indicate that owner-reported behavioral assessments and CDR scores may not fully capture subtle or preclinical cognitive changes, particularly in the earliest stages of disease. Alternatively, and consistent with observations in humans, some dogs may exhibit resilience to

underlying neuropathology, resulting in a dissociation between cognitive performance and pathological burden (Chew et al., 2025; de Vries et al., 2024; Latimer et al., 2019a).

Correlation analyses revealed that age was significantly associated with increased A β burden in the visual cortex, reinforcing age as the strongest risk factor for pathology (Figure 5). In contrast, CCDR scores were not significantly associated with neocortical A β burden (Figure 5). Astrocytic GFAP plasma measurement showed significant positive correlation with A β burden in both regions (Figure 8B and E). This finding parallels human studies demonstrating that an increase in plasma GFAP levels reflects astrocytic activation secondary to A β pathology (Pereira et al., 2021). Tau pathology was not significantly associated with age, CCDR scores, or plasma biomarkers in any regions (Supplemental Figures 7 and 9). This observation is consistent with previous findings indicated that tau pathology in aging dogs is limited with variable distribution (Abey et al., 2021a; Smolek et al., 2016; Wegiel et al., 1998; C.-H. Yu et al., 2011). In contrast, studies in AD have demonstrated strong associations between tau pathology and cognitive decline (Brier et al., 2016; Hanseeuw et al., 2019). Together, these results suggest that tau accumulation and distribution in aging dogs might represent a more heterogeneous or advanced stage process when compared to amyloid pathology, which could be readily detected by plasma biomarkers such as GFAP.

Within this cohort, dogs with intermediate Thal phases (2-3) and higher tau pathology frequently exhibited neocortical involvement; however, marked heterogeneity was observed. Some animals showed substantial amyloid deposition with minimal tau pathology, whereas others exhibited tau pathology in the presence of limited amyloid

burden. This variability suggests that additional pathogenic processes, including synucleinopathies, TDP-43 pathology, and vascular disease, may contribute to cognitive impairment and neuropathologic diversity in aging dogs, as they do in human neurodegenerative disease (Kapasi et al., 2017; Latimer et al., 2019a).

A major strength of this study is the systematic application of established human AD neuropathological frameworks to a naturally aging canine cohort. By adapting Thal phases for A β deposition, Braak-based regional assessment of tau pathology, and CERAD criteria for neuritic plaques, this work provides the first integrated clinico-pathologic evaluation of AD-like changes in companion dogs using criteria directly comparable to those employed in human studies. The use of client-owned dogs further enhances translational relevance, as these animals experience heterogeneous genetic backgrounds, shared human environments, and natural aging trajectories that are not captured in traditional laboratory models. Integration of standardized neuropathologic assessment with validated owner-reported cognitive measures and antemortem plasma biomarkers represents an additional strength, enabling evaluation of relationships among pathology, cognition, and peripheral markers within the same individuals.

Several limitations should also be considered. First, the cross-sectional, postmortem design precludes inference regarding the temporal sequence or causality of amyloid and tau pathology and their relationship to cognitive decline. Longitudinal studies incorporating serial cognitive assessments, biomarker measurements, and eventual neuropathologic evaluation will be essential to define disease trajectories in this model. Second, cognitive status was assessed using owner-reported instruments, which, while validated, may lack sensitivity to detect subtle or domain-specific

impairments, particularly in preclinical disease stages (Hargrave et al. 2024). Third, tau pathology was assessed using a pTau181-specific antibody, as commonly used human antibodies such as AT8 do not reliably label dog tau; therefore, the full spectrum of tau pathology in dogs may not be captured. Additionally, this study was not powered to assess the influence of sex, breed, body size, or lifespan variability, all of which may contribute to heterogeneity in aging trajectories and neuropathologic burden as only 14 of our samples were analyzed for biofluid biomarkers.

Despite these limitations, the strengths of this approach, particularly the use of standardized human neuropathological criteria in a naturally aging, translationally relevant species, support the value of the companion dog as a model for investigating early AD-related pathological processes.

3.6 Conclusion

Together, these findings support the validity of companion dogs as a naturally occurring model for AD-like pathology, particularly for investigating the earliest stages of disease. By integrating standardized human neuropathological frameworks with behavioral assessments and plasma biomarkers, our study bridges a critical the gap between veterinary and human aging research. Future studies should expand the characterization of mixed neuropathologies in dogs and further evaluate biomarker-pathology relationships using longitudinal and postmortem approaches. Such investigations will provide novel insights into the mechanisms that underly CCD and may inform translational strategies relevant to both veterinary and human neurodegenerative disease.

Chapter Four: Multiple Neuropathological Patterns Characterize Canine Cognitive Dysfunction Syndrome

Abdullatif Alsulami^{1,2}, Payton Shirley^{1,2}, Kapahi Kawai Puaa^{1,2}, Avery Lessard¹, Nikole Z. Madrid¹, Aidan Flannagan¹, Carolyn Dobkins¹, Stephanie McGrath^{2,3}, Caitlin S. Latimer⁴, Evan MacLean⁵, Julie A. Moreno^{1,2}

¹Department of Environmental & Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

²Brain Research Center, Colorado State University, Fort Collins, CO, USA

³Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

⁴Department of Laboratory Medicine and Pathology, University of Washington, Seattle, WA, USA

⁵Department of Psychology, College of Veterinary Medicine, University of Arizona, Tucson, USA

4.1 Abstract

Canine cognitive dysfunction (CCD) syndrome is a naturally occurring neurodegenerative disorder in aging dogs that shares clinical and neuropathological features with Alzheimer's disease (AD). While amyloid- β (A β) pathology has been well described in aged canines, the relationship between cognitive impairment, tau pathology, neuroinflammation and pathological heterogeneity within CCD remains poorly understood. Sixty-six canine brains were collected from privately owned aged dogs and processed for histopathological evaluation. Immunohistochemistry for A β , phospho-tau (T181, T217, T231), microglia and astrocytes were performed on frontal cortex and hippocampal sections. Cognitive status-based comparisons were conducted between cognitively impaired (CCD+) and cognitively intact (CCD-) dogs. Principal component analysis (PCA) followed by hierarchical clustering on principal components (HCPC) was used to characterize pathological heterogeneity in aged canines. CCD+ dogs showed significantly greater A β burden in both the frontal cortex and hippocampus compared with CCD- aged dogs. Meanwhile, tau pathology showed no significant differences between aged dogs with and without cognitive dysfunction. Neuroinflammatory markers demonstrated higher glial responses, with astrocytes significantly increased in the frontal cortex of CCD+ dogs, and microglial activation was more widespread elevated in both brain regions. Multivariate analysis identified three dimensions representing an amyloid-inflammatory axis and two epitope-specific tau axes with astrocytic responses. Unsupervised clustering characterized four distinct clusters, including low pathology, tau enriched and two mixed amyloid-inflammatory clusters. The amyloid- inflammatory dimension was independently associated with age

and cognitive impairment. Aged dogs with CCD show heterogeneous neuropathological patterns characterized by increased A β deposition, tau pathology and neuroinflammation. This heterogeneity within aged canines with CCD provide an opportunity to investigate specific pathological mechanisms that drive the progression. These findings further validate canines as a translational model of AD-related neurodegeneration.

4.2 Background

Neurodegenerative diseases, including Alzheimer's disease (AD), represent a major and rising public health concerns as global life expectancy increases. Aging is the primary risk factor for these disorders, which are characterized by the accumulation of misfolded proteins and a progressive increase in neuroinflammation, ultimately leading to synaptic dysfunction and neuronal loss (Hou et al., 2019).

Several rodent transgenic animal models have contributed to understanding the molecular mechanisms of neurodegenerative diseases. However, they lack the genetic diversity and pathological complexity of these diseases. Canines have substantial genetic homology with humans and display natural age-related cognitive decline and neuropathology (Olby et al., 2025; Ostrander et al., 2017). Their genetic diversity, rapid aging, and AD-like pathology position canines as a translational model for studying AD-like processes and testing therapeutic strategies (McGrath et al., 2025).

Canine cognitive dysfunction (CCD) syndrome is a naturally occurring neurodegenerative disease affecting aging dogs, typically over seven years of age (Szabó et al., 2016; Vikartovska et al., 2020). Clinically, CCD presents with behavioral

and cognitive impairments that closely resembles human dementia. Disease severity is commonly assessed using validated behavioral surveys such as the Canine Dementia Scale (CADES) and the Canine Cognitive Dysfunction Rating Scale (CCDR) (Madari et al., 2015; Salvin et al., 2011). Neuropathologically, CCD parallels the hallmarks of human AD, including the deposition of extracellular β -amyloid ($A\beta$) and hyperphosphorylated tau accumulation (Mihevc et al., 2019).

In AD, pathological hallmarks include $A\beta$ plaques, neurofibrillary tangles (NFT), synapse loss, and neuronal degeneration (Serrano-Pozo, Frosch, et al., 2011). Increasing evidence indicates that neuroinflammation has an early and potentially initiating role in disease progression (Heneka et al., 2013; Leng et al., 2021; Si et al., 2023). Glial activation in response to misfolded proteins may initially be protective; however, chronic activation drives astrocytes and microglia toward neurotoxic phenotypes that exacerbate protein aggregation and impair neuronal integrity (Heneka et al., 2013). Astrocytes and microglia are critical regulators of central nervous system homeostasis. Astrocytes maintain the blood–brain barrier, support metabolic and synaptic function, and modulate inflammatory signaling (Abbott et al., 2006; Manu et al., 2023). Microglia mediate immune surveillance, synaptic remodeling, and innate immune responses. Dysregulation of either cell type contributes to AD pathology and has been increasingly implicated in CCD (Hines et al., 2023; Thomsen et al., 2021). Chronic aggregation of $A\beta$ or tau drives both astrocytes and microglia into dysfunctional states characterized by cytokine release, oxidative stress, and impaired clearance of misfolded proteins (Hemali & Maniatis, 2015; Guan et al., 2022; Kim et al., 2024).

The histopathological standard for investigating CCD includes the assessment of A β plaques, tau pathology and elevated neuroinflammatory markers (Schütt et al., 2016b). This approach presumes a singular pathology pattern across all cognitively impaired dogs (Ferrer, 2024; Schmidt et al., 2015). However, evidence suggests the presence of pathological heterogeneity among individuals affected by AD and related dementia (Abdelnour et al., 2022; Duara & Barker, 2022a; Habes et al., 2020), raising the question of whether those dogs have a single or multiple pathological patterns compared to normal aging dogs. The histopathological heterogeneity in dogs affected by CCD has not yet been established.

In this study, we leveraged the canine model to bridge the translational gap in AD research through integration group based pathological comparisons with multivariate approach. We first quantified regional A β burden, tau pathology at multiple phosphorylation sites, and astrocytic and microglial activation. we then used a multivariate approach followed by hierarchical clustering to identify patterns of pathological heterogeneity across the overall cohort.

4.3 Methodology

Sample collection and preparation

Sixty-six canine brains were obtained from privately owned dogs with owner consent after euthanasia and necropsy. The brains were collected at the James L. Voss Veterinary Teaching Hospital at Colorado State University. Following collection, the brains were fixed in 10% neutral buffer formalin for at least 72 hours. After fixation, the brains were processed using a tissue processor (Leica TP 1020) and embedded in

paraffin wax using a tissue embedder (Leica EG 1160). Tissue blocks containing the cortex were cut from the frontal lobe and hippocampus blocks were selected based on the most representative area available. Using a microtome (ThermoFisher HM1030), sections were cut at 5 μ m and mounted on charged slides. We included canines that lacked the comorbidities of brain tumors or epilepsy.

Immunohistochemistry Staining

Immunohistochemical staining was performed to visualize various neurological markers, including Glial Fibrillary Acidic Protein (GFAP) (DAKO Cat. #20334, 1:400), Iba1 (Abcam 5076, 1:400), S100 β (Abcam 41428, 1:750), Phospho-Tau (Thr181) Monoclonal Antibody (Thermo Fisher MN1050, 1:200), Phospho-Tau-T217 Rabbit pAb (Abclonal AP1233, 1:200), Phospho-Tau-T231 Rabbit mAb (Abclonal AP0053, 1:200) and A β 1-16 (BioLegend 803014, 1:500). Sections were dewaxed in xylene and rehydrated through graded ethanol followed by antigen retrieval in boiling 0.01M sodium citrate for 20 minutes at 95°C. Endoperoxides were removed by incubating sections in a 0.3% hydrogen peroxide and water solution for 30 minutes. To block non-specific labeling, sections were treated with 10% horse serum in Tris-A with 2% BSA (bovine serum albumin) and 2% Triton-X in Tris buffered saline (TBS). Sections were then incubated overnight at 4°C with primary antibodies diluted in Tris-A with 2% BSA. After washing with Tris-A with 2% BSA, sections were incubated for one hour with their corresponding biotinylated secondary antibodies (goat anti-rabbit, horse anti-mouse or donkey anti-goat) diluted in 10% horse serum in Tris-A with 2% BSA at 1:250. Slides were counterstained with hematoxylin, rinsed in bluing reagent, dehydrated through graded ethanol followed by xylene, and mounted with mounting media. Coverslips were

applied using One cover glass per slide. Brightfield scans were acquired using Vectra Polaris Quantitative Pathology Imaging System (Akoya Biosciences) at 40x objective magnification.

Quantification of pathological markers

Quantitative analysis was performed using Qupath software (version 0.6.0) (Bankhead et al., 2017b; Nüesch et al., 2025) and Halo (v3.4.2986; Indica Labs). Briefly, after the acquisition of all scans, regions of interest (ROI) corresponding to grey matter were manually annotated. A machine learning object classifiers were trained to identify positive cells for each stain/antibody separately. Cell counts were normalized to area (cells/mm²) for each marker. Only grey matter was included in the cell counting. For amyloid-beta burden quantification, an area-based quantification approach was applied using the HALO Area Quantification module to calculate the percentage area occupied by positive staining within each ROI. Statistical Analysis

Group-based statistical comparisons were conducted on a subset of dogs that have complete clinical cognitive assessment data (n = 42). These analyses compared cognitively impaired dogs (CCD+) to cognitively normal aged controls (CCD-). Multivariate analyses were performed on the full cohort of dogs with available neuropathological data (n = 66), which included the subset used for group comparisons.

Prior to conducting group comparisons, normality was assessed using the Shapiro-Wilk test, and Levene's test was performed to check for homogeneity of variance. For group comparisons, two-sample t-tests were used where assumptions of

normality and homogeneity were met and Mann-Whitney test when normality assumption was violated.

The primary objective of the multivariate analysis was to characterize pathological heterogeneity in aged dogs and to identify pathologically distinct patterns. Initially, latent dimensions underlying variability in neuropathological and neuroinflammatory measures were identified using FactoMineR package (Lê et al., 2008). Subsequently, these dimensions were used as input for unsupervised clustering to classify dogs into pathology-driven subgroups.

First, principal component analysis (PCA) was conducted on regional measures of A β burden, tau pathology at multiple phosphorylation sites, and neuroinflammatory markers to perform dimensionality reduction. All variables were centered and scaled to unit variance prior to PCA. The first three dimensions, selected based on eigenvalues and scree plot, were retained for further analysis. Missing values were imputed before running PCA using a principal component approach in the missMDA package (Josse & Husson, 2016), with three components to balance variance capture and stability of the model.

Second step involved applying unsupervised clustering of the PCA-derived scores was used to classify dogs into sub-groups. Agglomerative hierarchical clustering was implemented using Ward's minimum variance method and Euclidean distance, which minimized within-cluster variance at each iteration. The optimal number of clusters was determined by majority consensus among multiple indices in the NbClust package (Charrad et al., 2014) and by visual inspection of the dendrogram. Hierarchical clustering on principal components (HCPC) assigned cluster ownership and

characterized clusters based on the original variables. Clusters were described using quantitative descriptors, including v-test statistics, to identify variables that significantly distinguished each cluster from other clusters and the overall cohort.

To evaluate the independent effects of age and cognitive status to pathological variation, multiple linear regression models were fitted with principal dimension scores as dependent variables and age and CADES score as predictors. All statistical analyses were performed using GraphPad Prism (version 10.1.2) and R studio (version 4.4.1) with statistical significance set at $p < 0.05$ for all analyses

4.4 Results

It is well documented that A β pathology is present in aging canines, and its burden is age (Pop et al., 2012; Prpar Mihevc & Majdič, 2019a). Previous findings indicate that amyloid pathology in aged canines follows a pattern similar to Thal's criteria. In this study, differences in A β burden were evaluated between cognitively healthy dogs, as determined by clinical diagnosis and CADES scores, and cognitively impaired dogs over seven years of age diagnosed with CCD. Cognitively impaired dogs exhibited a significantly higher A β burden in both the frontal cortex and hippocampus compared to age-matched controls (Figure 1). However, tau pathology, assessed at three phosphorylation sites, did not correspond with the clinical distinction between cognitively declined and normally aging dogs (Figure 2).

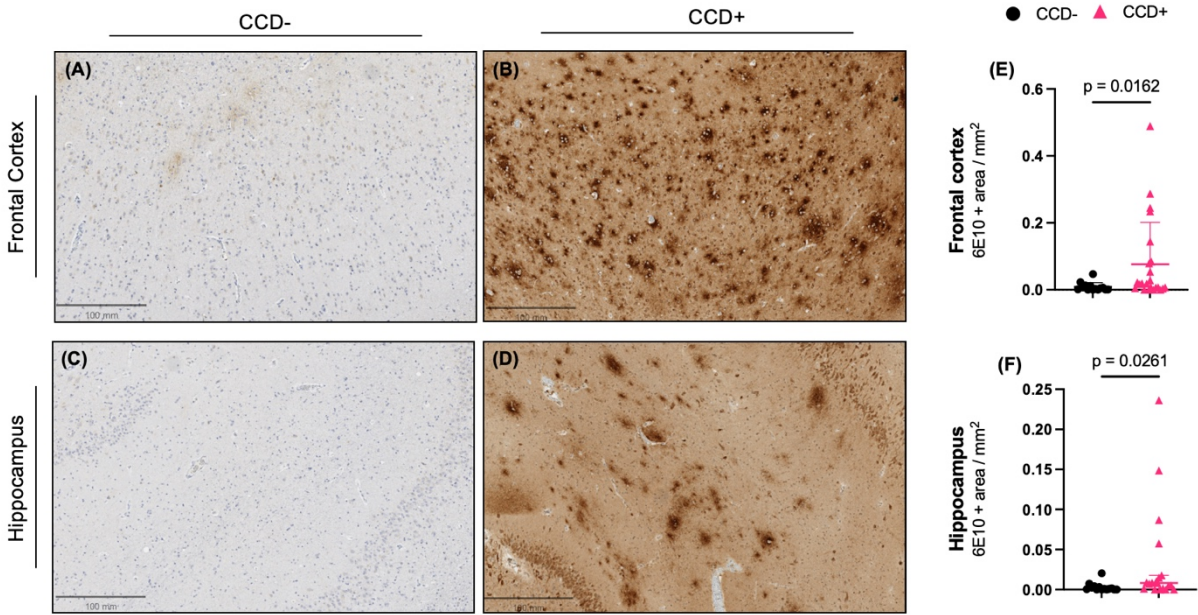


Figure 1: Increased burden of amyloid- β ($A\beta$) deposits in canines with cognitive dysfunction. (A–D) Representative immunohistochemical staining demonstrating $A\beta$ deposition in aged dogs without cognitive dysfunction (CCD-; A, C) and dogs clinically diagnosed with cognitive dysfunction (CCD+; B, D). Scale bars, 100 μ m. (E–F) Quantification of $A\beta$ burden (6E10-positive area per mm^2) in the frontal cortex (E) and hippocampus (F). CCD+ dogs display significantly increased $A\beta$ accumulation compared with CCD- dogs in both regions. Data are shown as individual dogs with mean $\pm$ standard deviation (SD), for panel E, and median $\pm$ interquartile range (IQR), for panel F.

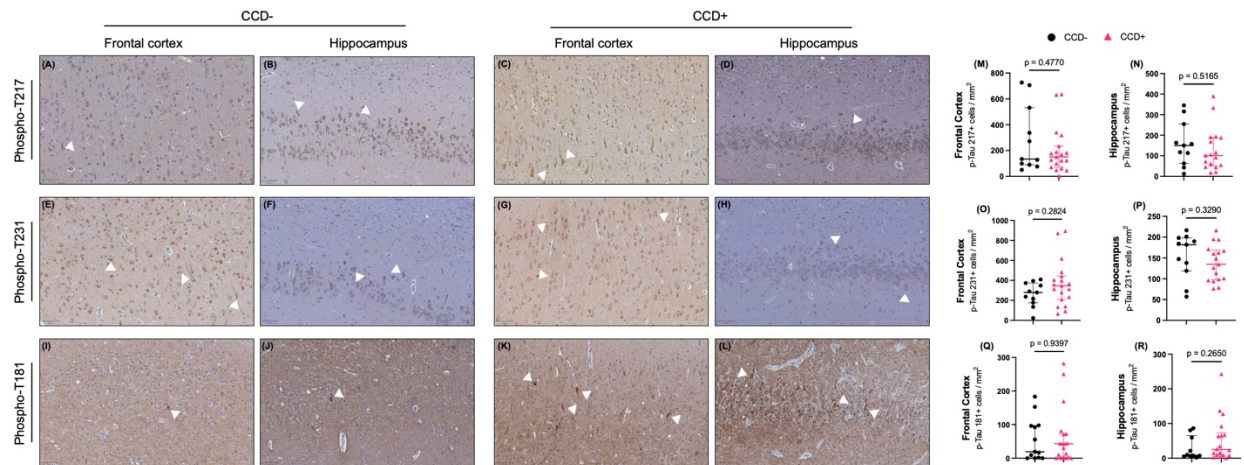


Figure 2. Phosphorylated tau (T217, T231, T181) burden in aged dogs with and without cognitive dysfunction (A–D) Representative immunohistochemical staining of phosphorylated tau at T217 in the frontal cortex (A, C) and hippocampus (B, D) of CCD- (A, B) and CCD+ (C, D) aged dogs. (E–H) Representative staining for phosphorylated tau at T231 in the frontal cortex (E, G) and hippocampus (F, H) of CCD- (E, F) and CCD+ (G, H) dogs. (I–L) Representative staining of phosphorylated tau at T181 in the frontal cortex (I, K) and hippocampus (J, L) of CCD- (I, J) and CCD+ (K, L) dogs. Scale bars, 100 μ m in all panels. (M–R) Quantification of p-T217+ (M, N), p-T231+ (O, P), and p-T181+ (Q, R) cell densities (positive cells/mm²) in the frontal cortex and hippocampus showed no statistically significant differences. Individual dogs are plotted with median $\pm$ interquartile range (IQR).

To determine whether cognitive impairment in aging dogs is associated with elevated neuroinflammation, astrocytic and microglial markers were quantified in the frontal cortex and hippocampus. Dogs with cognitive dysfunction (CCD+) demonstrated a significant increase in astrocyte number, as indicated by higher GFAP and S100b positive cells, in the frontal cortex compared to age-matched control dogs (CCD-)

(Figure 3). No group differences in astrocytic number were observed in the hippocampus. Microglial activation was more widespread and elevated in both the frontal cortex and hippocampus of CCD+ dogs, indicating both region-specific and generalized inflammatory responses associated with cognitive dysfunction.

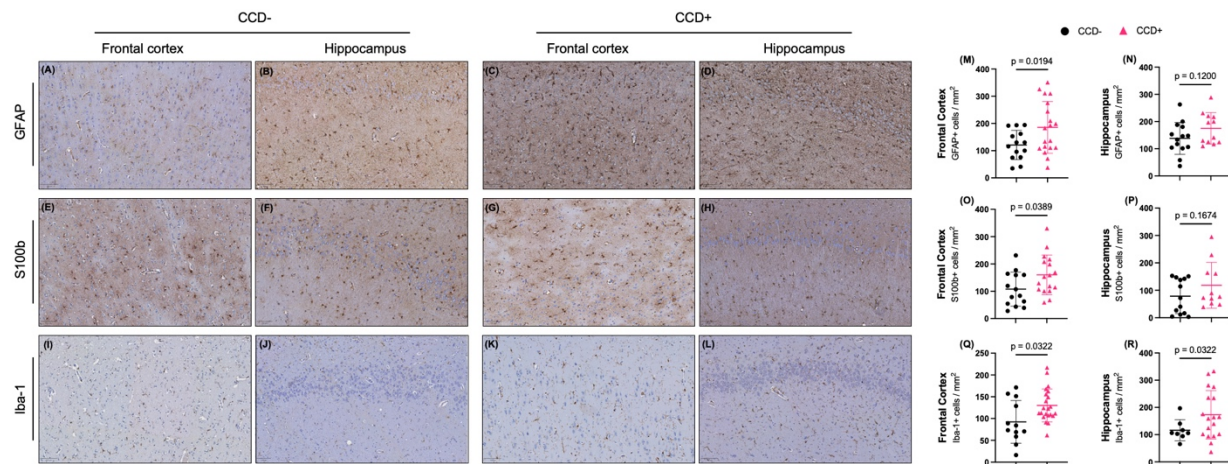


Figure 3. Astrocytic and microglial activation in aged dogs with and without cognitive dysfunction. (A–D) Representative immunohistochemical staining of GFAP in the frontal cortex (A, C) and hippocampus (B, D) of CCD- (A, B) and CCD+ (C, D) dogs. (E–H) Representative staining for S100b in the frontal cortex (E, G) and hippocampus (F, H). (I–L) Representative Iba-1 immunostaining of microglia in the frontal cortex (I, K) and hippocampus (J, L). (M–R). Scale bars, 100 μm in all panels. Quantification of GFAP+ (M, N), S100b+ (O, P), and Iba-1+ (Q, R) cell densities (cells/mm²) in the frontal cortex and hippocampus. CCD+ dogs had significantly higher values for frontal cortex GFAP+ (M), frontal cortex S100b+ (O), frontal cortex Iba-1+ (Q), and hippocampal Iba-1+ (R). Individual animals are plotted with mean $\pm$ standard deviation (SD), for panels M, N, O, Q, and R and median $\pm$ interquartile range (IQR), for panel P.

The PCA model identified three dimensions that together explained 51.7% of the variance in the data. The loadings of the variables to the three dimensions are shown in Table 1. The first dimension accounted for 19.2% of the variance and mostly defined by A β burden in the frontal cortex and hippocampus, and microglial activation in both regions. Astrocytic S100 β measures also loaded positively from both regions, whereas phospho-tau showed mixed directionality (positive significant loading of hippocampal phospho-tau181, negative loading of cortical phospho-tau181 hippocampal phospho-tau217). The loadings indicate that this dimension reflects an amyloid-inflammatory response axis.

The second dimension accounted for 18.2% of the variance and was mostly driven by tau associated measures and astrocytic response, with strong positive loadings of phospho-tau181, tau217, and astrocytic S100 β in both regions along with positive loadings by hippocampal phospho-tau231. In contrast, hippocampal A β and astrocytic GFAP loaded negatively, suggesting that the axis captures tau pathology with astrocytic activation. The third dimension accounted for 14.3% of the variance and was mostly driven by phospho-tau217 and astrocytic GFAP activation in both regions. Together, dimensions 2 and 3 indicate an epitope specific variation of tau pathology coupled with astrocytic responses, which are distinct compared to the first dimension.

Variables	Dimension 1 Coefficient (contribution%)	Dimension 2 Coefficient (contribution%)	Dimension 3 Coefficient (contribution%)
β -amyloid Frontal cortex	0.704 (18.42)*	0.012 (0.005)	0.154 (1.20)
β -amyloid Hippocampus	0.740 (20.39)*	-0.342 (4.58)*	-0.068 (0.23)
P-tau181 Frontal cortex	-0.362 (4.86)*	0.407 (6.51)*	0.155 (1.20)
P-tau181 Hippocampus	0.329 (4.03)*	0.671 (17.70)*	0.021 (0.02)
P-tau217 Frontal cortex	0.075 (0.21)	0.287 (3.24)*	0.680 (23.12)*
P-tau217 Hippocampus	-0.409 (6.22)*	0.273 (2.92)*	0.460 (10.61)*
P-tau231 Frontal cortex	0.229 (1.9)	-0.327 (4.19)*	-0.565 (15.98)*
P-tau231 Hippocampus	-0.236 (2.06)	0.259 (2.63)*	-0.381 (7.27)*
GFAP Frontal cortex	0.343 (4.3)*	-0.163 (1.05)	0.718 (25.84)*
GFAP Hippocampus	-0.024 (0.02)	-0.636 (15.87)*	0.438 (9.63)*
S100b Frontal cortex	0.404 (6.07)*	0.654 (16.78)*	-0.106 (0.56)
S100b Hippocampus	0.399 (5.91)*	0.772 (23.40)*	-0.066 (0.22)
Iba1 Frontal cortex	0.571 (12.14)*	-0.093 (0.33)	0.207 (2.14)
Iba1 Hippocampus	0.598 (13.28)*	-0.136 (0.73)	-0.195 (1.91)

Table 1. Variables coefficients for PCA dimensions. Values represent the coefficients and contributions of each variable for the three retained PCA dimensions. Positive and negative signs represent the directionality of the variable loadings within each dimension. Asterisks (*) denote variables that are significantly associated to each dimension.

We applied hierarchical agglomerative clustering on the three dimensions from the PCA model (Figure 4). Cluster 1 harbored 19% of the total dogs included in the study (N= 13), cluster 2 had 56% (N=37), cluster 3 and 4 each a 12.2% of total dogs (n= 8). Variation of clusters was based on HCPC standardized comparison, with positive values indicative of over-representation within a cluster and negative values indicate

under-representation relative to the overall cohort. The clusters descriptors are shown in Table 2. Cluster 1 was characterized significantly by higher phospho-tau217 in both regions, and cortical phospho-tau181. This cluster represents tau only pathology with increased astrocytic response in the hippocampus. Dogs in this cluster were significantly under-represented by hippocampal A β and microglia, and cortical phospho-tau231. This cluster manifest as tau enriched, with lower A β compared to overall cohort.

Cluster 2 had the largest number of dogs in one cluster, and it was characterized by broad under-representation across multiple pathological measures, including A β and phospho-tau. It represents a low pathology pattern where all pathological measures are significantly lower compared to overall cohort.

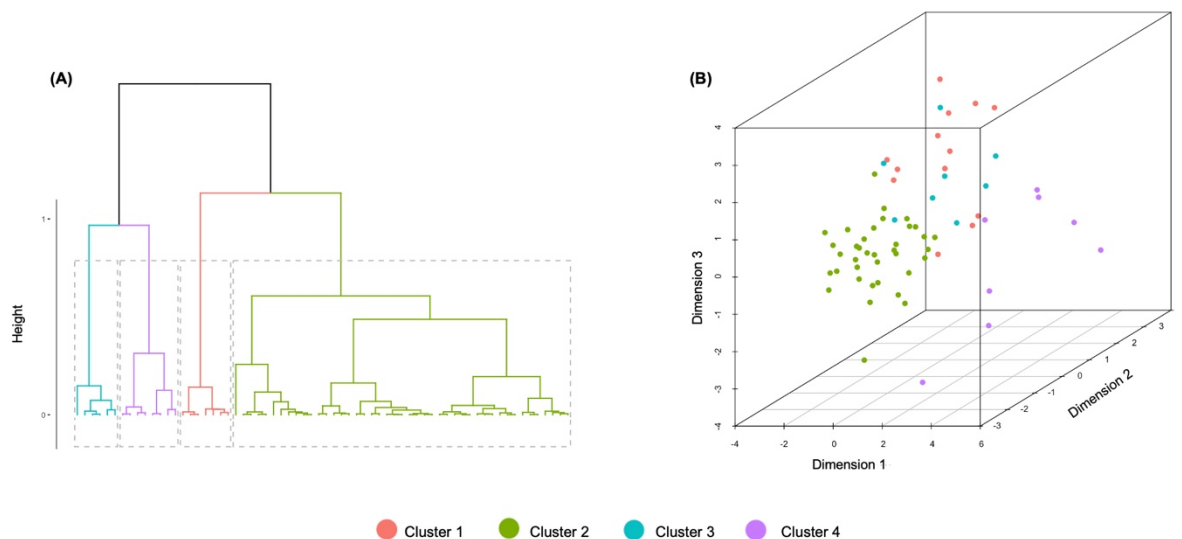


Figure 4. Hierarchical clustering of aged dogs based on PCA pathology measures. (A) Dendrogram based on hierarchical clustering on principal components (HPCP), with individual dogs depicted on the x-axis (each terminal branch represents an individual dog from the cohort). The y-axis depicts dissimilarities, where shorter distances along

the axis indicate greater similarities. (B) Three-dimensional space representation of the first three dimensions retained from the PCA model. Dots represent individual dogs and are colored according to the cluster assignment.

Cluster 1						
Variable	v.test	Mean in category	Overall mean	SD in category	Overall SD	p.value
Phospho-tau217 Hippocampus	4.359505	218.1334077	124.5016145	1.13E+02	85.75837686	1.30E-05
Phospho-tau181 Frontal Cortex	3.48582	98.37866388	48.42209732	5.42E+01	57.22398348	4.91E-04
Phospho-tau217 Frontal Cortex	3.403865	323.8512	184.8533662	2.39E+02	163.0519877	6.64E-04
S100 β Hippocampus	3.022258	145.1643794	92.8209417	5.72E+01	69.15461225	2.51E-03
β -amyloid Hippocampus	-2.121906	0.00303137	0.02647865	6.81E-03	0.04412215	3.38E-02
Iba1 Hippocampus	-2.429241	111.0963722	163.4067737	3.65E+01	85.98206444	1.51E-02
Phospho-tau231 Frontal Cortex	-3.015596	231.5225735	371.946173	1.21E+02	185.933394	2.56E-03
Cluster 2						
β -amyloid Hippocampus	-2.481820	0.01445411	0.02647865	0.02620124	0.04412215	1.307132E-02
β -amyloid Frontal Cortex	-2.990789	0.02297593	0.06370993	0.04784968	0.12403087	2.782573E-03
S100 β Hippocampus	-4.964574	55.12066220	92.82094170	37.21229225	69.15461225	6.885214E-07
S100 β Frontal Cortex	-4.464676	109.37234140	143.17655996	45.39700912	68.95082872	8.018999E-06
Phospho-tau181 Hippocampus	-3.941178	21.03497715	38.67543093	19.95821941	40.76076212	8.108258E-05
Phospho-tau217 Frontal Cortex	-3.569497	120.94251376	184.85336624	74.19967479	163.05198773	3.576669E-04
GFAP Frontal Cortex	-2.825561	114.31658648	138.37353897	52.60518309	77.53440344	4.719789e-03
Cluster 3						
GFAP Frontal Cortex	5.411719	278.5070594	138.373539	55.22050437	77.53440344	6.24E-08
GFAP Hippocampus	3.291708	204.2207499	142.6841761	39.900881	55.97571719	9.96E-04
Iba1 Frontal Cortex	3.267251	182.4146309	121.6083073	56.31558186	55.72549908	1.09E-03
β -amyloid Hippocampus	3.036405	0.07122202	0.02647865	0.05028574	0.04412215	2.39E-03
β -amyloid Frontal Cortex	3.025911	0.18905242	0.06370993	0.07651946	0.12403087	2.48E-03
Phospho-tau217 Frontal Cortex	2.24549	307.131716	184.8533662	159.6395975	163.0519877	2.47E-02
Iba1 Hippocampus	2.232191	227.5058185	163.4067737	121.3127513	85.98206444	2.56E-02
Phospho-tau231 Hippocampus	-2.395409	115.7484239	151.1847226	31.55278995	44.29517247	1.66E-02
Cluster 4						
S100 β Hippocampus	4.334491	192.9295822	92.8209417	75.29700798	69.15461225	1.46E-05
β -amyloid Frontal Cortex	3.757603	0.21936133	0.06370993	0.23972974	0.12403087	1.72E-04
S100 β Frontal Cortex	3.667637	227.6340507	143.17656	72.04931311	68.95082872	2.45E-04
Phospho-tau181 Hippocampus	3.399588	84.95410664	38.67543093	65.29680622	40.76076212	6.75E-04
β -amyloid Hippocampus	3.323368	0.07545059	0.02647865	0.06572206	0.04412215	8.89E-04
Iba1 Hippocampus	2.330538	230.3299369	163.4067737	118.3504838	85.98206444	1.98E-02
Phospho-tau217 Hippocampus	-2.670506	48.01552893	124.5016145	42.45113121	85.75837686	7.57E-03
GFAP Hippocampus	-3.110602	84.53327276	142.6841761	41.07095927	55.97571719	1.87E-03

Table 2. Cluster-specific pathological patterns identified by HCPC. Variables contributing significantly to each cluster were identified using HCPC v-tests. For each variable, the

v-test statistic, cluster-specific mean, overall mean, standard deviation, and associated p-value are reported. Positive v-test values denote enrichment within the cluster while negative values denote relative depletion compared to the overall cohort.

Cluster 3 was distinguished by significant over-representation of neuroinflammatory markers, both astrocytic and microglial responses in both regions. Also, A β burden in both regions were higher in this cluster compared to the overall cohort. Phospho-tau was significantly over-represented within this cluster at cortical tau217 site, and under-represented at hippocampal tau231.

Cluster 4 was characterized by significant over-representation of A β burden and astrocytic S100 β response in both regions, along with increased hippocampal Phospho-tau181 and microglial activation. Phospho-tau181 and astrocyte GFAP response in the hippocampus were significantly under-represented in this cluster.

Although Clusters 3 and 4 both showed elevated A β and inflammation associated markers relative to the overall cohort, they differed in the glial activation and tau site patterns (Cluster 3 had a broader astrocytic GFAP/microglial elevation in both regions with cortical phospho-tau217 while Cluster 4 had astrocytic S100 β /hippocampal microglial activation with hippocampal phospho-tau181), supporting the presence of more than one A β -glial inflammatory axis within the cohort.

To assess whether pathological dimensions from the PCA model were associated with age and cognitive scores, multiple linear regression models were fitted with dimensions scores as the outcome and age and CADES scores as the predictors (Figure 5 and Table 3). Dimension 1 showed significant association with age (β = 0.14,

95% CI [0.009 - 0.288], p-value= 0.036) and CADES scores (β = 0.03, 95% CI [0.016 - 0.046], p-value= 0.0001), indicating that this dimension of pathological variation is associated with aging and cognitive impairment. In contrast, dimensions 2 and 3 had no significant associations with age and CADES scores.

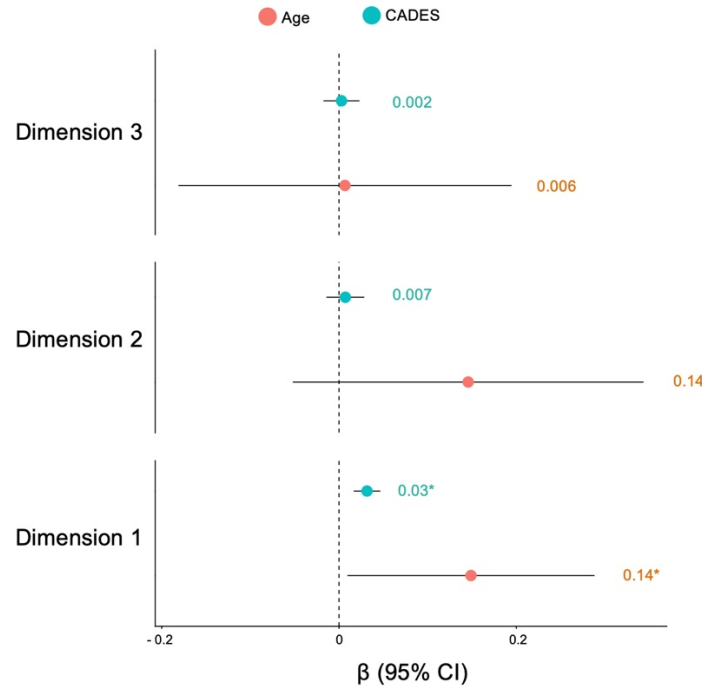


Figure 5. Associations of age and cognitive impairment with principal component dimensions. Forest plot showing regression coefficients (β) and 95% confidence interval (CI) from separate regression models evaluating the association of age and CADES score with each of the first three principal components. The dashed vertical line represents no association ($\beta = 0$). Significant associations were observed for dimension 1 for both predictors while no significant associations were detected for Dimensions 2 and 3. Asterisks (*) denote $p < 0.05$.

Dimension	Predictor	Estimate	95% CI	Std. Error	t value	p value
Dim1	Age	0.14886	0.009 - 0.288	0.068923	2.16	0.036994
Dim1	CADES	0.031535	0.016 - 0.046	0.007452	4.232	0.000136
Dim2	Age	0.14572	-0.052 - 0.343	0.09784	1.489	0.144
Dim2	CADES	0.00709	-0.014 - 0.028	0.01058	0.67	0.507
Dim3	Age	0.006706	-0.181 - 0.194	0.092864	0.072	0.943
Dim3	CADES	0.00282	-0.017 - 0.023	0.010041	0.281	0.780

Table 3. PCA-dimension one is associated with age and cognitive dysfunction. Separate multiple linear regression models were fitted for each principal component dimension, with age and CADES score included as predictors. Regression coefficients (β), 95% confidence interval (CI), standard errors, t statistics, and corresponding p-values are reported.

4.5 Discussion

Transgenic rodent models have been extensively utilized to study AD-related neuropathological features; however, canines offer a unique advantage as a model for AD research (Ford et al., 2025; Kitazawa et al., 2012). Their shared environmental factors with humans and the natural pathological progression of CCD closely mirror the human condition, positioning them as a valuable avenue for studying AD (Head, 2013). In this study, we investigated pathological heterogeneity by integrating cognitive status based neuropathological comparisons with a multivariate approach that includes misfolded protein burden and neuroinflammatory responses.

Consistent with previous studies, we found that A β deposition is more profound in dogs with behavioral signs of cognitive decline, Figure 1 (Ozawa et al., 2016; Pugliese et al., 2006). While total tau phosphorylation did not significantly differ between normal and cognitively impaired dogs, subtle increases in early phospho-tau181 immunoreactivity were observed in cognitively impaired dogs (Figure 2). Phospho-tau doesn't appear to follow the clinical distinction of cognitive score. Some cognitively impaired dogs show phospho-tau immunoreactivity while some cognitively healthy dogs do show phospho-tau. A similar, though less pronounced, inconsistency was observed with A β pathology, where some of the dogs that do have positive cognitive scores do not show amyloid pathology, and vice versa. This might suggest the presence of another key pathological factor that is either masking or driving cognitive decline.

Neuroinflammation plays a central role in AD pathogenesis (Liu et al., 2022; W. Zhang et al., 2023). Initially, astrocyte and microglia have critical protective responses to the insult, protein aggregation, but these cells transition to a more toxic phenotype as the number of activated cells increases and pathology progresses (Gao et al., 2023; Li et al., 2019; Miao et al., 2023; Zimmer et al., 2024). In this study, we evaluated glial activation in both the frontal cortex and hippocampus of dogs affected by CCD. We found that CCD dogs exhibited increased astrocyte and microglia activation in the frontal cortex, with only microglia number elevated in the hippocampus of cognitively declined dogs. These results underscore the critical role of glial cells in CCD pathology, corresponding to increased A β burden in both regions. Furthermore, the findings highlight the potential of CCD as a naturally occurring model for investigating AD pathogenesis, as it demonstrates heightened pathology and inflammation closely

resembling human AD. These findings reinforce the central role of glial cells in CCD pathology and support growing evidence that neuroinflammation is an integral component of disease-associated brain changes. However, cognitive status-based analyses alone masked substantial inter-individual variability.

To address this heterogeneity, we applied principal component analysis (PCA), followed by hierarchical clustering on principal components (HCPC), enabling identification of latent pathological dimensions and data-driven subgroups without predefined disease categories. PCA revealed three principal dimensions that captured distinct patterns of pathological variation. The first dimension reflected an amyloid–microglial inflammatory axis, while the second and third dimensions represented epitope-specific tau variation coupled with astrocytic responses. These findings indicate that amyloid-associated inflammation and tau-associated astrocytic processes constitute separable biological axes within the aging canine brain.

Unsupervised clustering of these dimensions identified four pathological patterns. The largest cluster was characterized through broad under-representation of amyloid, tau, and neuroinflammatory markers, consistent with relatively a low-pathology brain. The prevalence of CCD is estimated to be 14–35% of aging dogs, and the presence of a large low-pathology cluster in our cohort aligns with the observation that only a smaller subset of aged dogs develop CCD-associated neuropathological changes (Azkona et al., 2009; Salvin et al., 2010).

The remaining clusters represented distinct pathological patterns rather than a single disease continuum. One cluster was characterized by enrichment of phospho-tau217 in both the frontal cortex and hippocampus, elevated cortical phospho-tau181,

and increased astrocytic S100 β , but comparatively low amyloid burden and reduced microglial activation. This pattern suggests a tau-enriched phenotype driven by specific phosphorylation events rather than generalized tau accumulation. Another cluster was defined by robust astrocytic and microglial activation along with elevated A β burden, with only modest tau involvement, consistent with an amyloid-associated inflammatory pattern. A fourth cluster exhibited elevated amyloid burden, increased astrocytic S100 β , and hippocampal phospho-tau181, along with hippocampal microglial activation, but reduced phospho-tau217 and GFAP expression, indicating a mixed-pathology pattern. Notably, clusters with similar pathological components differed in regional distribution and relative intensity, highlighting the complexity of pathological aging in dogs.

Regression analyses provided additional insight into the clinical relevance of these pathological dimensions. Only the first PCA dimension, defined primarily by A β burden and neuroinflammation, was independently associated with both age and cognitive impairment (CADES score). In contrast, dimensions driven largely by tau epitope-specific variation and astrocytic responses were not independently associated with age or cognitive status. Even though multiple pathological patterns are present in the aging canine brain, these results suggest that only a subset is directly associated with cognitive decline.

Together, these results parallel emerging evidence from human neurodegenerative disease research indicating that AD and related dementias comprise multiple biological heterogeneous groups rather than a single homogeneous disease (Avelar-Pereira et al., 2023; Habes et al., 2020). The presence of several pathological patterns in aged dogs supports the translational relevance of the canine model for

studying AD and related dementias. Recognizing and characterizing this heterogeneity is critical for improving interpretation of neuropathological findings and guiding future studies aimed at crossing pathological patterns to longitudinal cognitive outcomes.

4.6 Conclusion

AD remains a significant global health challenge, and progress in understanding its natural progression and pathological complexity is constrained by the limited availability of translational models that fully recapitulate the human condition. CCD syndrome, a naturally occurring age-related cognitive decline in dogs, offers a unique opportunity to address this translational gap. The present findings indicate that aging dogs with cognitive dysfunction exhibit key pathological features relevant to human AD, including A β accumulation, neuroinflammatory responses, and early phospho-tau alterations.

Importantly, the data demonstrate that pathological aging in dogs is heterogeneous, encompassing distinct patterns of amyloid burden, tau phosphorylation, and glial activation rather than a single uniform disease trajectory. This heterogeneity parallels emerging concepts in human AD and underscores the value of the canine model for studying biologically diverse neurodegenerative pathways. Future studies incorporating additional pathological markers, such as α -synuclein and other misfolded proteins, as well as longitudinal cognitive assessments, will be essential for further refining the role of CCD Syndrome as a translational model for Alzheimer's disease and related dementias.

Chapter Five: Glial Cells Morphological Assessment and Relationship to Amyloid Aggregates

Abdullatif Alsulami^{1,2}, Payton Schneider^{1,2}, Stephanie McGrath^{2,3}, Gisele Patron¹ and Julie A. Moreno^{1,2*}

¹Department of Environmental & Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

²Brain Research Center, Colorado State University, Fort Collins, CO, USA

³Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO, USA

5.1 Abstract

Alzheimer's disease (AD) is characterized by the progressive deposition of amyloid-beta ($A\beta$) plaques, tau neurofibrillary tangles, and neuroinflammatory changes involving microglia and astrocytes. Although glial activation is increasingly recognized to have an integral role in AD pathogenesis, the spatial and morphological relationship between glial cells and $A\beta$ plaques remains incompletely understood, especially in naturally aging models. Canine cognitive dysfunction (CCD) provides a valuable translational model as dogs naturally develop cognitive decline and AD-like neuropathological features. In this study, $A\beta$ plaques associated glial responses were evaluated in aged dogs with and without cognitive impairment to determine whether microglia and astrocytes interact with $A\beta$ pathology. Using immunofluorescent and digital pathology approaches, morphological and spatial analyses were used to characterize glial cell associations with plaques and to determine whether plaque burden corresponded with local neuroinflammatory changes. In addition, a smaller treatment cohort of aged dogs with cognitive dysfunction was examined to explore whether repurposed therapeutic candidates, including rapamycin, cannabidiol, and trazodone, were associated with differences in $A\beta$ plaque burden and glial cell density.

5.2 Background

Alzheimer's disease (AD) is the most common cause of dementia and represents a major global health and economic burden (Duan et al., 2025; Lanctôt et al., 2024; Tay et al., 2024). With more than 50 million people living with dementia worldwide, the need to better understand the mechanisms underlying AD pathogenesis is critical. Despite extensive research efforts, effective treatments remain limited, particularly those capable of slowing or modifying disease progression (Passeri et al., 2022; Yiannopoulou & Papageorgiou, 2020). For this reason, repurposing drugs with already established safety and pharmacokinetic profiles has emerged as a promising strategy, as it could accelerate the identification and evaluation of promising therapeutic candidates (Cummings et al., 2025; Grabowska et al., 2023).

Neuropathologically, AD is characterized by the accumulation of misfolded proteins, particularly amyloid-beta ($A\beta$) and hyperphosphorylated tau, together with prominent neuroinflammation (Y. Chen & Yu, 2023; Long & Holtzman, 2019). $A\beta$ deposition is thought to be among the earliest pathological events in AD and has been associated with early and mild cognitive changes, whereas tau pathology is more closely linked to neurodegeneration and clinical progression (C. Zhang et al., 2025; H. Zhang et al., 2021). In addition to these classical proteinopathies, neuroinflammation is now recognized as a central component of AD pathogenesis. Activated microglia and astrocytes are consistently observed in association with amyloid plaques and neurofibrillary tangles pathology, highlighting their importance in the progression of disease (Heneka et al., 2015; Kamila et al., 2025; Kinney et al., 2018).

Microglia, the resident immune cells of the central nervous system, play critical roles in debris clearance, synaptic remodeling, and phagocytosis of aggregated proteins, including A β (Qin et al., 2023). Similarly, astrocytes contribute to maintenance of brain homeostasis, but shift to a reactive phenotype with the progressive nature of AD and are frequently enriched around plaques (H. Y. Kim et al., 2026). Both cell types undergo substantial morphological and functional changes in response to their surrounding microenvironment (Garland et al., 2022). Importantly, glial responses around plaques are not only a secondary response, but may influence plaque remodeling, local inflammatory signaling, and neuronal injury. While complex inflammatory mechanisms have been proposed to modulate AD progression, important gaps remain in our understanding of how glial cells interact with A β within the local tissue environment (Bouvier et al., 2016; Deczkowska et al., 2018; Huang et al., 2021; Serrano-Pozo, Mielke, et al., 2011b). Characterizing these plaque-associated glial responses, including their morphology and spatial association, may provide important insight into disease severity and progression.

Several therapeutic strategies have been explored to reduce A β burden and modify AD-related pathology. However, repeated failures and serious adverse events of anti-amyloid therapies in human clinical trials have shifted attention toward repurposing existing compounds with established safety profiles (Forlenza & Barbosa, 2025; Yiannopoulou et al., 2019). Among these, cannabidiol (CBD), rapamycin, and trazodone have emerged as promising candidates. Rapamycin has been widely studied as a longevity inducer compound and acts through inhibition of the mechanistic target of rapamycin (mTOR) pathway (Zerdka et al., 2025). In transgenic models, rapamycin

treatment has been reported to reduce plaque burden, improve cognitive performance, and preserve blood-brain barrier integrity (Halloran et al., 2012; Majumder et al., 2011; Van Skike et al., 2018). CBD has also been proposed as a therapeutic candidate in dementia because of its antioxidant, anti-inflammatory, and neuroprotective properties, including modulation of endocannabinoid-related pathways (Atalay Ekiner et al., 2022; Hickey et al., 2024; Singh et al., 2023). Likewise, preclinical studies suggest that trazodone may mitigate cognitive dysfunction and neurodegenerative changes (Gonçalo & Vieira-Coelho, 2021). Although these treatments are promising, they have not been adequately investigated in a naturally aging model that more closely reflects the spontaneous development of AD-like pathology.

While transgenic animal models have provided important mechanistic insight, they do not fully recapitulate the spontaneous, age-associated nature of AD (Z.-Y. Chen & Zhang, 2022; Pádua et al., 2024). In contrast, aged companion dogs develop canine cognitive dysfunction (CCD), a naturally occurring syndrome that shares multiple behavioral, pathological, and molecular features with human AD (McGrath et al., 2025; Vitturini et al., 2025). Dogs with CCD exhibit cognitive and behavioral impairments that can be assessed clinically using owner-based assessment tools such as the Canine Dementia Scale (CADES) (Madari et al., 2015). Neuropathologically, CCD is associated with A β deposition, tau-related pathology, and increased neuroinflammation, supporting the value of this model for translational studies of aging and dementia (Alsulami et al., 2025b; Habiba et al., 2021; Hines et al., 2023).

In this study, we evaluated glial cells association with A β plaques in cognitively intact and cognitively impaired dogs to determine its pathological relevance. We further

examined the relationship between A β plaques burden and neuroinflammation through morphological and spatial analyses of glial cells. Finally, in a smaller cohort of dogs treated with rapamycin, CBD, or trazodone, we assessed amyloid pathology and its intersection with neuroinflammatory changes. Together, this work aims to improve the importance of plaque-associated glial responses in a naturally occurring model of AD and to explore the therapeutic potentials of repurposed treatment in age-related cognitive decline.

5.3 Methodology

Dogs Recruitment

Canine brains were obtained from privately owned dogs with owner consent after euthanasia and necropsy. The brains were collected at the Veterinary Teaching Hospital at Colorado State University. Following collection, the brains were fixed in 10% neutral buffer formalin for at least 72 hours. After fixation, the brains were processed using a tissue processor (Leica TP 1020) and embedded in paraffin wax using a tissue embedder (Leica EG 1160). Tissue blocks containing the pre-frontal cortex (middle frontal gyrus) were cut from the frontal lobe based on the most representative area available. Using a microtome (ThermoFisher HM1030), sections were cut at 5 μ m and mounted on charged slides. We excluded dogs that presented with comorbidities that could impact their cognitive function, such as brain tumors or epilepsy.

Immunohistochemistry

Immunohistochemical staining was performed to visualize various markers, including Glial Fibrillary Acidic Protein (GFAP) (DAKO Cat. #20334, 1:400), Iba1

(Abcam 5076, 1:400), S100 β (Abcam 41428, 1:250), and A β 1-16 (BioLegend 803014, 1:500). Sections were dewaxed in xylene and rehydrated through graded ethanol followed by antigen retrieval in boiling 0.01M sodium citrate for 45 minutes at 240°C. To block non-specific labeling, sections were treated with 5% horse serum in Tris buffered saline (TBS) and 2% Triton-X. Sections were then incubated overnight at 4°C with primary antibodies diluted in TBS with 2% horse serum. After washing with (TBS), sections were incubated for one hour with their corresponding biotinylated secondary antibodies (goat anti-rabbit, horse anti-mouse or donkey anti-goat) diluted in 10% horse serum in TBS with 2% horse serum at 1:500. Brightfield and florescence scans were acquired using Vectra Polaris Quantitative Pathology Imaging System (Akoya Biosciences) at 20x objective magnification.

Glial Cells Morphological Analysis

Morphological analysis was performed to characterize glial cell structure in plaque-associated and non-plaque regions (J. Kim et al., 2024; Young & Morrison, 2018). For each dog, two representative images were selected from each domain. The non-plaque domain consisted of areas without visible amyloid plaque involvement, whereas the plaque-associated domain consisted of regions containing amyloid plaques and adjacent glial cells. In the non-plaque domain, each image included a minimum of five cells for analysis. For the plaque-associated domain, a similar number of cells were collected when possible; however, the final number of analyzable cells varied depending on plaque abundance and the number of clearly identifiable cells present within or near each plaque. This sampling approach was designed to capture cells from both pathological contexts while maintaining consistency across animals and domains.

Image preprocessing was carried out following the general workflow (Young & Morrison, 2018). Briefly, images were first prepared to optimize single-cell morphological assessment, included cleaning steps to remove background interference and improve cell visibility, followed by image binarization to convert cells into suitable objects for downstream morphology analysis. These preprocessing steps were necessary to standardize cell representation and ensure that subsequent measurements reflected cell morphology rather than variability in staining intensity or image background.

After preprocessing, individual cells were isolated from each image for single-cell analysis. Only cells with enough visible morphology and processes were selected for further evaluation. Cells that were incomplete, poorly resolved, excessively overlapped with neighboring cells, or otherwise unsuitable for reliable segmentation were excluded during image preparation. This step was important to ensure that the extracted morphological data represented individual glial cells with interpretable branching architecture and overall cellular shape.

Binarized images containing cells went through batch processing generating single cropped cells and their corresponding skeletons as described in the microglial morphology pipeline (J. Kim et al., 2024). Skeletonization reduces the cell to a one-pixel-wide representation of its branching structure while preserving the overall topology of the processes. This approach enables quantification of features related to branching organization and process architecture, such as branch number, junctions, endpoints, branch length, and other measures reflecting the branching complexity and extent of cellular arborization. Skeleton-based measurements were used to capture structural

differences in glial morphology that may reflect changes in activation state or local microenvironment.

In addition to skeleton analysis, fractal analysis was performed on each isolated cell to evaluate geometric complexity and shape-related properties. Fractal analysis provides quantitative descriptors of cellular ramification and structural irregularity that are not fully captured by simple size-based or branch-count measurements alone (Sedivy et al., 1999). These features are particularly useful when assessing glial cells, as both microglia and astrocytes can undergo marked morphological remodeling in response to pathological insult, such as the presence of plaques. Together, skeleton and fractal analyses provided complementary information on cell branching, complexity, and overall morphological organization.

Morphological outputs from each cell were exported and assembled into a single analysis pipeline in R studio for subsequent processing. Data generated from skeleton and fractal analyses were tidied, reformatted, and merged so that measurements from each individual cell could be linked back to its corresponding dog and domain. Cells from the same dog and the same domain were then grouped together into unified datasets. This organization allowed morphology to be examined at the single-cell level while preserving the biological structure of the dataset across animals and pathological regions. Following data assembly, additional cleaning steps were applied to remove variables that were not informative or were not consistently measured across cells. Variables were reviewed for quality and suitability prior to downstream analysis, and only morphology features retained after these preprocessing steps were included in the

final dataset. The resulting dataset therefore consisted of cleaned single-cell morphological measurements derived from all dogs included in the study.

Glial Cells Spatial Analysis

Spatial analysis was performed to characterize the localization of glial cells in relation to amyloid plaques (Mi et al., 2020; W. Zhang et al., 2025). Pixel classifiers were trained in Qupath to identify amyloid plaque signal across tissue sections (Bankhead et al., 2017b; Nüesch et al., 2025). After plaque detection, classifier outputs were converted into plaque annotations, and each detected plaque was separated into an individual object for plaque-level spatial assessment. This allowed each plaque to serve as a discrete spatial reference for downstream analyses. To identify glial cells, separate object classifiers were trained for microglia and astrocytes. These classifiers were applied independently so that each cell population could be detected and analyzed separately within the same tissue context. Once plaque annotations and glial detections were established, Qupath spatial analysis tools were used to calculate the distance from each detected cell to the nearest plaque and only cells located within 100 μm of the nearest plaque were retained. Negative distance values were interpreted as cells located within plaque boundaries, whereas positive distance values represented cells located outside the plaque at increasing distances from the plaque edge. Cells with distances below 0 μm were therefore classified as inside plaques, while cells with distances between 0 and 100 μm were classified as plaque-associated but external to the plaque core. This workflow enabled quantitative evaluation of glial localization within and around amyloid plaques and provided a plaque-centered measure of the local inflammatory microenvironment.

Glial Cells Coverage of Amyloid-Beta Plaques

To quantify glial coverage of amyloid plaques, pixel classification-based analysis was performed in Qupath. Separate pixel classifiers were generated for amyloid plaques and for glial markers. The amyloid plaque pixel classifier was trained to accurately detect plaque-positive regions within tissue sections, whereas the glial pixel classifiers were developed to capture the full labeled glial area, including both soma and cellular processes, rather than cell detection alone. This approach was used to quantify the extent of glial signal occupying plaque regions. Amyloid plaques were first identified using the plaque pixel classifier. The resulting plaque-positive regions were then converted into annotations, and individual plaques were separated into distinct plaque objects for downstream measurement. This step allowed each plaque to be treated as an independent region of interest for coverage analysis.

Following plaque annotation, separate glial pixel classifiers were applied for microglia and astrocytes. In contrast to object-based cell detection, these classifiers were designed to identify the total positive glial area within plaque annotation, thereby capturing the full extent of glial morphology present within plaque regions. Only glial signal located within plaque annotations was included in the analysis.

For each plaque annotation, the percentage of area occupied by glial-positive signal was calculated and used as a measure of plaque coverage by microglia or astrocytes. This plaque-restricted area fraction provided an estimate of the extent to which glial processes and cell bodies infiltrated or occupied amyloid plaque regions. Coverage measurements were then used to assess microglial and astrocytic association with amyloid plaques.

Results

Microglia and astrocytes show descriptive morphological differences

To evaluate glial morphological changes in relation to A β plaques and cognitive impairment, we assessed astrocytes and microglia shape and branching descriptors across plaque-free and plaque-associated domains in cognitively intact and cognitively impaired dogs. As shown in Figure 1, both astrocytes and microglia show morphological differences depending on their plaque environment.

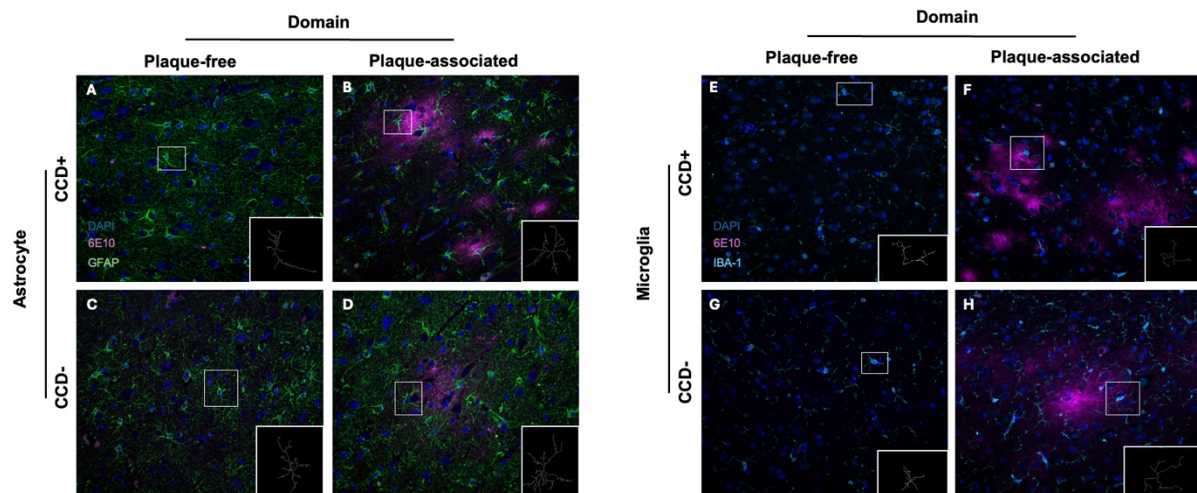


Figure 1. Representative images of astrocyte and microglial cells in plaque-free and plaque-associated domains from cognitively impaired (CCD+) and cognitively intact aged dogs (CCD-). Panels A–D show astrocytes in CCD+ plaque-free (A), CCD+ plaque-associated (B), CCD– plaque-free (C), and CCD– plaque-associated (D) regions. Panels E–H show microglia in CCD+ plaque-free (E), CCD+ plaque-associated (F), CCD– plaque-free (G), and CCD– plaque-associated (H) regions. White boxes indicate representative cells selected for single-cell morphological analysis with insets representing skeletonized cells.

Microglial cells in dogs with cognitive dysfunction exhibit activated phenotype

Microglial activation is increasingly recognized as an important component of cognitive decline and neurodegeneration (Fan et al., 2024). To determine whether cognitive status in aged dogs is associated with altered microglial morphology, morphological features were compared between CCD+ and CCD- dogs (Figure 2). Microglia from dogs with cognitive dysfunction showed significantly reduced branching complexity (Figure 2, panels A-H), including lower branches, junctions, endpoint voxels, junction voxels, slab voxels, triple points, and lower total pixels when compared to cognitively intact aged dogs. In contrast, average branch length was increased in CCD+ microglia. These changes observed indicate that microglial cells in CCD+ dogs are in a more activated state with less branching.

Parameters related to cell size and compactness also showed changes between the two groups (Figure 2, panels I-P). CCD+ microglia had lower area, perimeter, width and height of the bounding rectangle, maximum span across the hull, and mean radius. Circularity was not significantly changed between the two groups, whereas the coefficient of variation for all radii was increased in CCD+ microglia, suggesting more irregularities of microglial shape in cognitively impaired dogs. Together, these findings indicate that microglia in cognitively impaired dogs adopt a less structurally complex and less ramified morphology, consistent with structural changes associated with disease status (Vidal-Itriago et al., 2022).

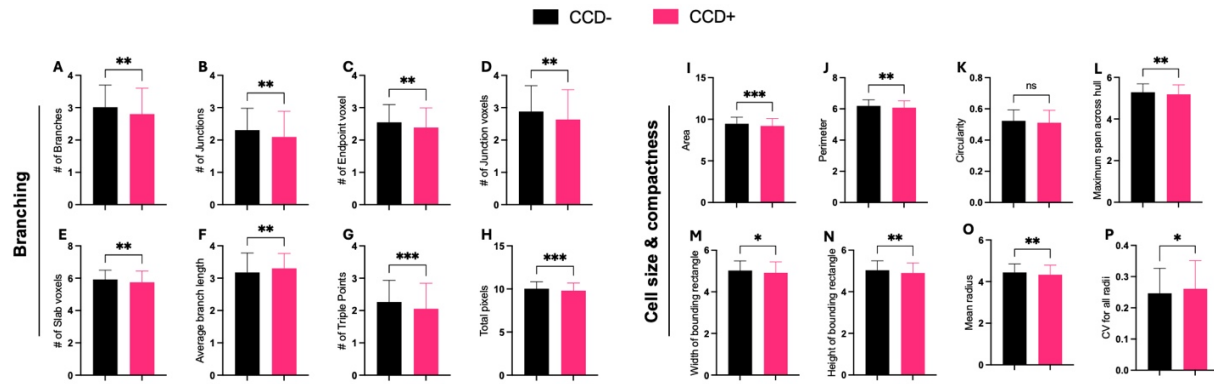


Figure 2. Microglial cells from cognitively impaired dogs exhibit reduced branching complexity and altered cell shape. Quantitative comparison of microglial morphological features between cognitively intact aged dogs (CCD-, black) and dogs with canine cognitive dysfunction (CCD+, pink). Branching-related features are shown in panels A–H, including number of branches (A), number of junctions (B), number of endpoint voxels (C), number of junction voxels (D), number of slab voxels (E), average branch length (F), number of triple points (G), and total pixels (H). Cell size and compactness features are shown in panels I–P, including area (I), perimeter (J), circularity (K), maximum span across hull (L), width of bounding rectangle (M), height of bounding rectangle (N), mean radius (O), and coefficient of variation (CV) for all radii (P). Microglia from CCD+ dogs showed significantly reduced branching complexity across multiple parameters and alterations in cell size and shape, consistent with a less ramified morphology in cognitively impaired animals. Data are presented as mean $\pm$ SD. Statistical significance is indicated as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; ns, not significant.

Plaque association has limited effects on microglial morphology compared to cognitive status

The aggregation of A β plaques has been associated with local inflammatory microenvironment (Fixemer et al., 2025). To determine whether plaque proximity is associated with distinct microglial morphology, microglial cells from plaque-associated and non-plaque microenvironments were compared (Figure 3). None of the microglial branching features differ significantly between the two domains (Figure 3 A-H). Similarly, most parameters associated with cell size and compactness were unchanged between plaque-associated and non-plaque microglia, including area, perimeter, circularity, maximum span across the hull, width of the bounding rectangle, mean radius, and coefficient of variation for all radii. The only significant difference observed was in the height of the bounding rectangle, which suggests that microglial cells associated with non-plaque microenvironment are more vertically spread (Figure 3, panel N). Overall, these results show that in this cohort, cognitive status is associated with broader microglial morphological differences than plaque proximity alone.

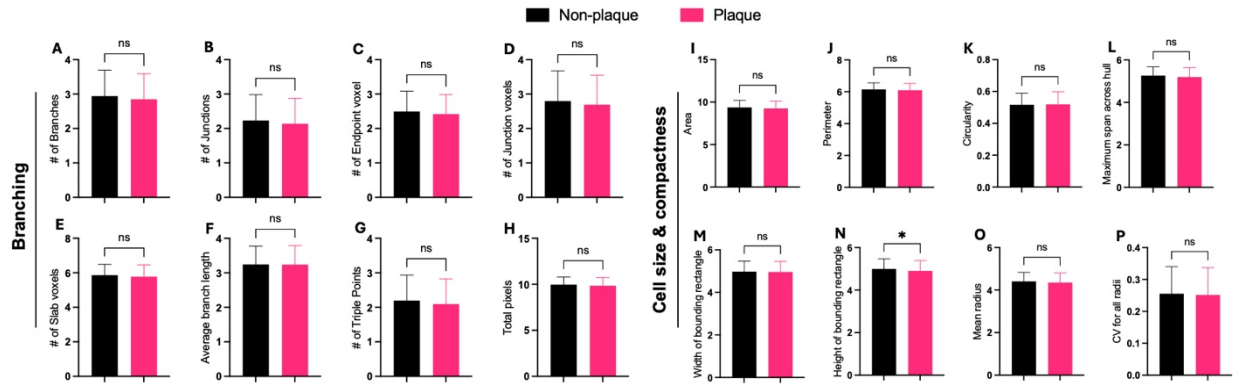


Figure 3. Plaque proximity has limited effects on microglial morphology. Quantitative comparison of microglial morphological features between non-plaque (black) and plaque-associated (pink) domains. Branching-related features are shown in panels A–H, including number of branches (A), number of junctions (B), number of endpoint voxels (C), number of junction voxels (D), number of slab voxels (E), average branch length (F), number of triple points (G), and total pixels (H). Cell size and compactness measures are shown in panels I–P, including area (I), perimeter (J), circularity (K), maximum span across hull (L), width of bounding rectangle (M), height of bounding rectangle (N), mean radius (O), and coefficient of variation (CV) for all radii (P). Most microglial features did not differ significantly between plaque-associated and non-plaque domains, with the exception of height of the bounding rectangle (N), suggesting that plaque proximity alone had limited effects on overall microglial morphology in this cohort. Data are presented as mean $\pm$ SD. * $p < 0.05$; ns, not significant.

Astrocytes in cognitively impaired dogs show selective reductions in branching features

Astrocytes mediated responses are a well-established component of AD, although their morphological alterations may differ from those of microglia, they still provide information on neuroinflammatory states and neurotoxicity (Cheng et al., 2019). To assess whether astrocytes morphology differ based to cognitive status, we applied a similar approach (Figure 4). Astrocytes from cognitively impaired dogs had significantly fewer branches, fewer junctions, and fewer junction voxels compared to cognitively intact aged dogs, with some other branching-related variables, including endpoint voxels, slab voxels, average branch length, triple points, and total pixels, not significantly different between groups (Figure 4 A-H).

Measures of astrocytic size and compactness were not significantly different between CCD+ and CCD- dogs (Figure 4, panels I-P). These findings indicate that astrocytes in dogs with cognitive dysfunction represent selective changes in cellular branching organization, while overall cell size and compactness relatively preserved.

Plaque-associated astrocytes show marked morphological remodeling

Reactive astrocytes are frequently enriched around amyloid plaques and may undergo substantial structural changes in response to the local pathological environment. To evaluate whether plaque association influences astrocyte morphology, plaque-associated and plaque-free astrocytes were compared (Figure 5). Plaque-associated astrocytes showed significantly reduced branching complexity, including fewer branches, junctions, endpoint voxels, junction voxels, and triple points compared to non-plaque astrocytes (Figure 5, panels A-H). Also, plaque-associated astrocytes

showed increased average branch length, increased slab voxels, and increased total pixels. Changes in cell parameters were also prominent, with plaque-associated astrocytes having greater cell area, perimeter, width of the bounding rectangle, maximum span across the hull, and mean radius compared to astrocytes in non-plaque regions (Figure 5, panels I-P). Together, these findings indicate that plaque-associated astrocytes adopt a distinct remodeled morphology characterized by reduced fine branching but greater overall size and process extension.

Astrocyte and microglia undergo morphological remodeling in the aged canine brain; however, the pattern differs by cell type. Microglial morphology showed that CCD+ dogs have an overall reduced branching and smaller cell dimensions. In contrast, astrocytes showed more pronounced differences according to plaque domain, with plaque-associated astrocytes displaying a distinct enlarged and remodeled morphology despite reduced fine branching. These findings support that glial responses in canine cognitive dysfunction are influenced by both disease status and the local amyloid plaque microenvironment.

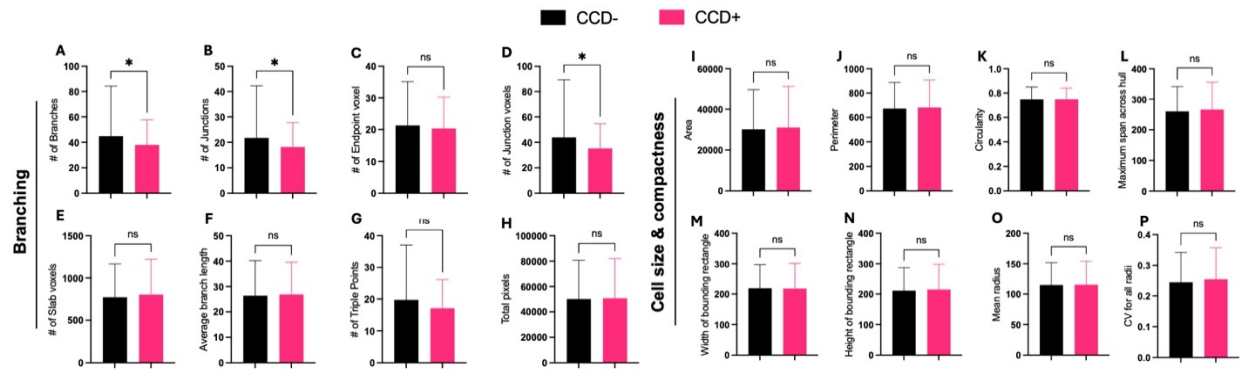


Figure 4. Astrocytes from cognitively impaired dogs show selective reductions in branching features. Quantitative comparison of astrocyte morphological features between cognitively intact aged dogs (CCD–, black) and dogs with canine cognitive dysfunction (CCD+, pink). Branching-related parameters are shown in panels A–H, including number of branches (A), number of junctions (B), number of endpoint voxels (C), number of junction voxels (D), number of slab voxels (E), average branch length (F), number of triple points (G), and total pixels (H). Cell size and compactness parameters are shown in panels I–P, including area (I), perimeter (J), circularity (K), maximum span across hull (L), width of bounding rectangle (M), height of bounding rectangle (N), mean radius (O), and coefficient of variation (CV) for all radii (P). Astrocytes from CCD+ dogs exhibited selective reductions in branching-related features, particularly branches, junctions, and junction voxels, whereas measures of cell size and compactness were not significantly altered. Data are presented as mean $\pm$ SD. * $p < 0.05$; ns, not significant.

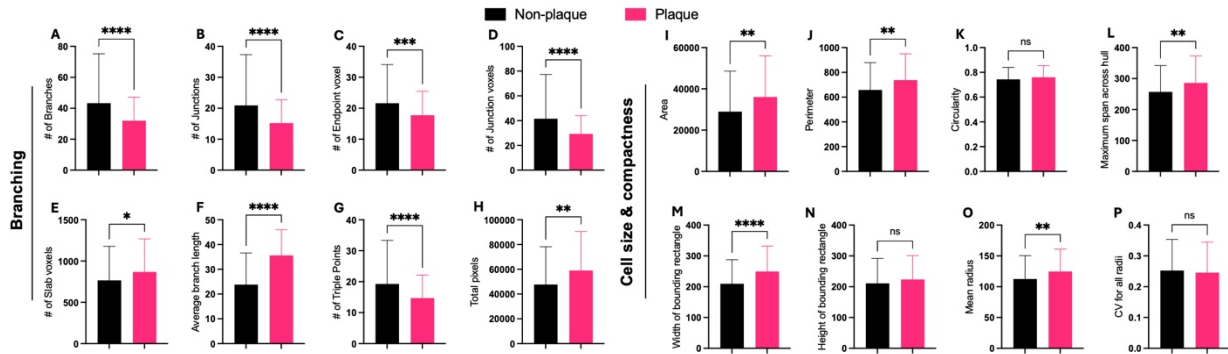


Figure 5. Plaque-associated astrocytes exhibit marked morphological remodeling. Quantitative comparison of astrocyte morphological features between non-plaque (black) and plaque-associated (pink) domains. Branching-related features are shown in panels A–H, including number of branches (A), number of junctions (B), number of endpoint voxels (C), number of junction voxels (D), number of slab voxels (E), average branch length (F), number of triple points (G), and total pixels (H). Cell size and compactness features are shown in panels I–P, including area (I), perimeter (J), circularity (K), maximum span across hull (L), width of bounding rectangle (M), height of bounding rectangle (N), mean radius (O), and coefficient of variation (CV) for all radii (P). Plaque-associated astrocytes showed significantly reduced fine branching complexity together with increased cell area, perimeter, span, and radius, indicating a distinct enlarged and remodeled morphology in the local plaque microenvironment. Data are presented as mean $\pm$ SD. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; ns, not significant.

Morphological features in aged dogs show distinct glial cells phenotypes

Glial cells morphology is highly multidimensional, and individual features alone may not fully represent the structural variation present across cells. To better characterize this variation, principal component analysis (PCA) was performed on the retained glial morphological features followed by k-means clustering (Figure 6). First, we included 27 structural morphology measures related to cellular branching and complexity in PCA models corresponding to microglial or astrocytic cells separately. In microglial cells, correlation analysis across features showed strong relationships between branching-related variables and cellular complexity variables, indicating that several measurements capture related aspects of microglial structure (Figure 6, panel A). The PCA loading heatmap showed that the retained three principal components separated branching, size, and complexity-related features across distinct axes of variation (Figure 6, panel B).

Using the three retained principal components, microglia cells were classified into six morphologically distinct clusters based on the silhouette method for k-means clustering. The cluster-specific heatmap demonstrated that these six clusters differed in their relative representation of branching features, cell size measures, and shape-related variables (Figure 6, panels C). The first cluster was characterized by an irregular microglial morphology, with reduced branching and smaller area, suggesting a relatively elongated and uneven cellular profile. The second cluster represented moderately ramified microglia. Cluster three showed stronger contributions from size and spread-related features, together with increased branching compared with Clusters one and two, suggesting larger, more territorially expanded and complex phenotype. The fourth

cluster harbored cells that had reduced branching but larger cell size, consistent with a more amoeboid-like morphology. Cluster five was characterized by fewer but longer processes and irregular cellular outlines, suggesting an elongated, rod-like morphology. The sixth cluster was associated with highly branched cells with smaller cell area, consistent with a compact, highly ramified microglial phenotype. The cluster plot further showed partial separation of these six microglial groups across principal component space, indicating the presence of multiple recurring microglial morphologies within the dataset rather than a single continuum of structural change (Figure 6, panel C).

To determine whether the identified microglial morphological clusters were associated with plaque proximity or cognitive status, cluster distributions were compared by plaque domain and cognitive status (Table 1). Across plaque-associated and non-plaque regions, the proportional representation of the six clusters was similar, and no significant difference in cluster distribution was detected by domain. Similarly, comparison of cluster representation between CCD+ and CCD- dogs showed no significant association between cognitive status and microglial cluster distribution. Although some shifts in cluster percentages were observed descriptively, these differences did not reach statistical significance. These findings indicate that while microglial cells display substantial morphological heterogeneity, the overall distribution of the identified cluster types was not significantly different by plaque domain or CCD status in our cohort.

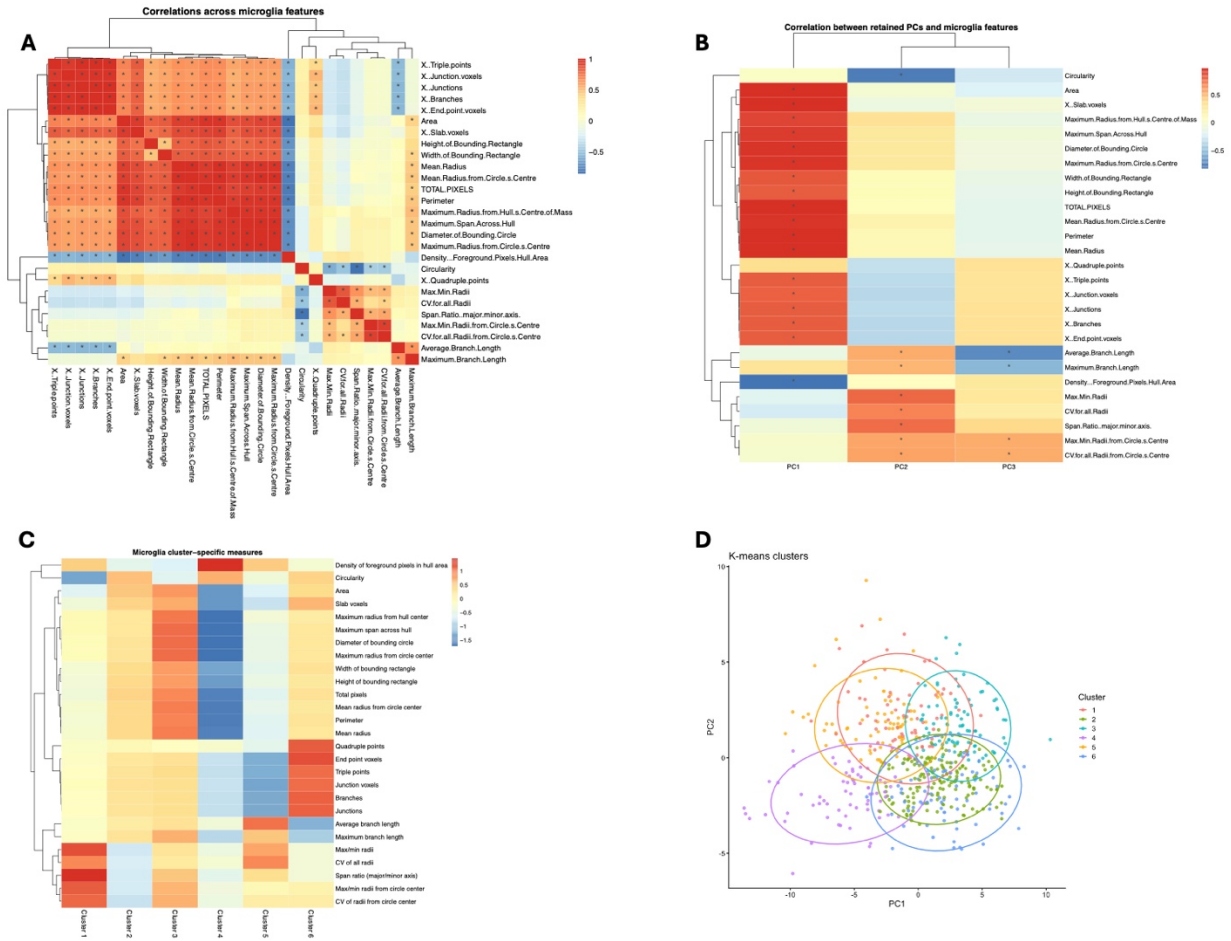


Figure 6. Microglial cells are morphologically heterogeneous with distinct structural clusters.

(A) Heatmap showing correlations among microglial morphological features. Features cluster into major groups corresponding to branching complexity, cell size, and compactness-related measures, indicating coordinated structural relationships across microglial morphology. Positive correlations are shown in red and negative correlations in blue, with hierarchical clustering illustrating feature grouping. (B) Heatmap of correlations between retained principal components (PC1–PC3) and individual

microglial morphological features. PC1 is strongly associated with cell size and overall structural extent, PC2 reflects branching-related complexity, and PC3 captures more subtle shape and proportional features. This dimensional reduction highlights the major axes underlying microglial morphological variability. (C) Heatmap of cluster-specific standardized feature values for microglia, illustrating the distinct morphological profiles that define each k-means cluster. Clusters represent recurring structural phenotypes characterized by differences in branching complexity, spatial extent, and shape-related properties, supporting the presence of heterogeneous microglial states within the aging canine brain. (D) Principal component analysis (PCA) plot showing k-means clustering of microglial cells in PC1-PC2 space. Each point represents a single cell, colored by cluster membership, with ellipses indicating cluster distributions. Clusters show partial separation but also overlap, reflecting continuous yet structured heterogeneity in microglial morphology rather than discrete, fully separated subtypes.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6
Domain						
Non-plaque	12%	29.36%	18.28%	10.80%	15.51%	14.40%
Plaque	15%	29.35%	12.50%	15.76%	12.50%	14.67%
CCD status						
CCD-	12%	32.36%	18.55%	10.18%	12.36%	14.91%
CCD+	14%	26.30%	14.07%	14.81%	16.67%	14.07%

Table 1. Distribution of microglial morphological clusters across plaque domains and cognitive status. Microglial cells were classified into six morphological clusters based on principal component analysis and k-means clustering. The relative distribution (%) of each cluster is shown for non-plaque and plaque-associated domains, as well as for dogs with negative (CCD-) and positive (CCD+) cognitive status. Cluster distributions were comparable between plaque and non-plaque regions and between CCD groups. Chi-square analysis revealed no significant association between cluster distribution and domain ($\chi^2 = 6.8837$, $df = 5$, $p = 0.2294$) or cognitive status ($\chi^2 = 8.1562$, $df = 5$, $p = 0.1478$).

Similar to microglia, astrocytic morphology is composed of multiple correlated structural features that may reflect different aspects of cellular remodeling. To characterize this variation, PCA was employed on the same variable that were used for microglia, followed by k-means clustering on the retained astrocytic principal components (Figure 7). Correlation analysis demonstrated strong relationships among branching and size-related astrocytic variables (Figure 7, panel A), while the PCA loading structure indicated that the retained components captured distinct axes of astrocytic morphological variation (Figure 7, panel B).

With three retained principal components, astrocytes were classified into four morphologically distinct clusters (Figure 7, panel C). The cluster-specific heatmap showed that these four groups differed in branching organization, size-related measures, and shape-associated features. The cluster distribution in principal component space demonstrated partial separation between groups, supporting the presence of recurring astrocytic morphologies across the dataset. These findings

indicate that astrocytes, like microglia, exhibit substantial morphological heterogeneity that can be interpreted as distinct structural subtypes.

The first cluster was characterized by highly branched cells, consistent with a more structurally complex astrocytic morphology. The second cluster represented larger cells with moderately increased branching features, suggesting expanded astrocytic morphology with relatively longer processes. The third cluster showed less extensive branching and a more compact morphology. The last cluster was characterized by more irregularly shaped cells, with elongated and asymmetrical cellular profiles.

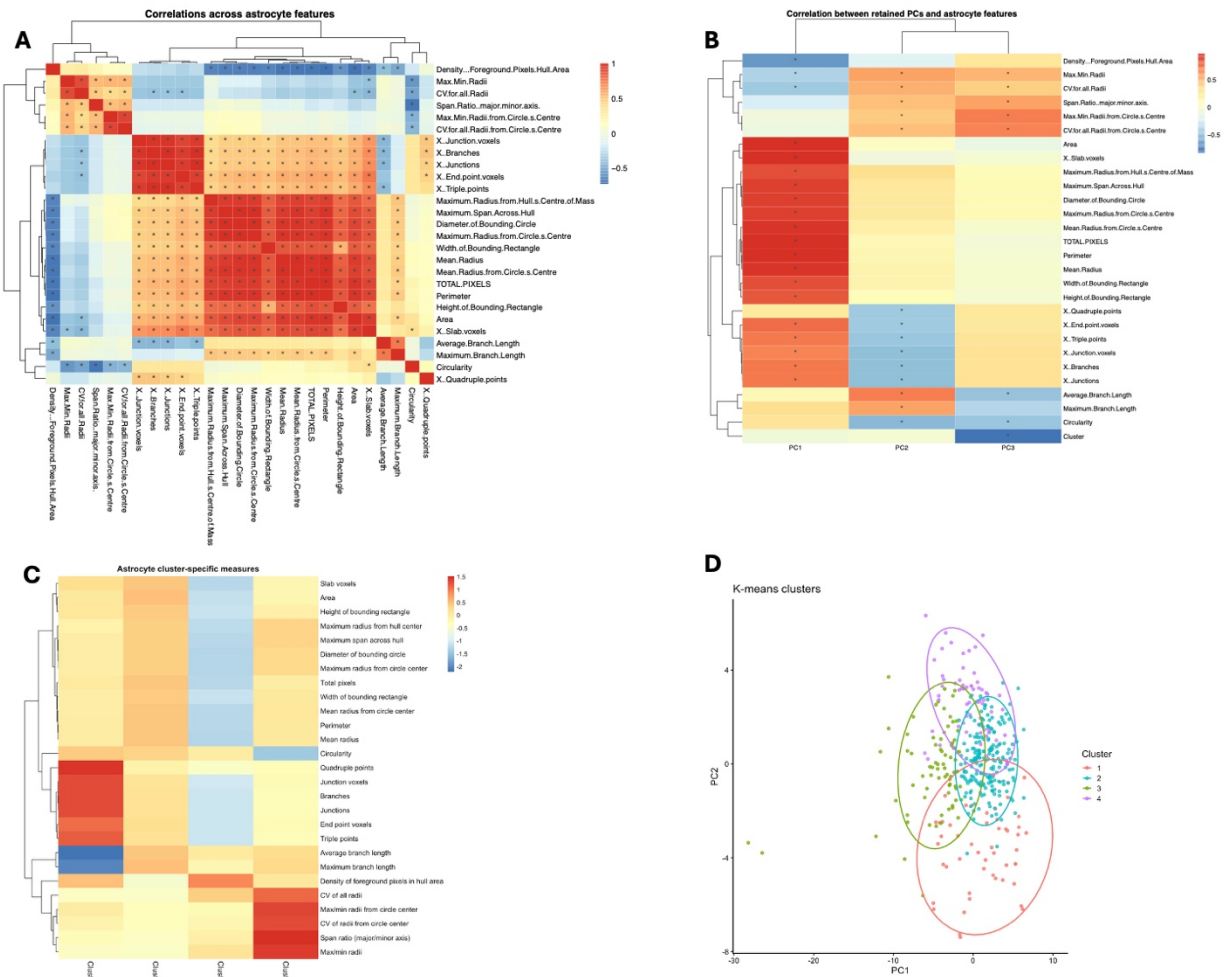


Figure 7. Morphologically distinct astrocytic population. (A) Heatmap showing pairwise correlations among astrocyte morphological features. Features group into major clusters reflecting branching complexity, cell size, and compactness-related properties, indicating coordinated structural relationships across astrocyte morphology. Positive correlations are shown in red and negative correlations in blue, with hierarchical clustering illustrating feature organization. (B) Heatmap of correlations between retained principal components (PC1–PC3) and astrocyte morphological features. PC1 is primarily associated with cell size and structural extent, PC2 captures branching complexity, and PC3 reflects shape-related and proportional features. These principal components summarize the major axes of astrocyte morphological variation. (C) Heatmap of cluster-specific standardized feature values for astrocytes, demonstrating distinct morphological profiles across k-means clusters. Each cluster represents a recurring astrocyte phenotype characterized by differences in branching, spatial extent, and shape, supporting the presence of heterogeneous astrocytic states in the aging canine brain. (D) Principal component analysis (PCA) plot showing k-means clustering of astrocytes in PC1–PC2 space. Each point represents a single cell colored by cluster assignment, with ellipses indicating cluster distributions. Clusters show partial separation with some overlap, reflecting continuous but structured heterogeneity in astrocyte morphology rather than fully discrete subtypes.

	Cluster 1	Cluster 2	Cluster 3	Cluster 4
Domain				
Non-plaque	16.78%	45.72%	20.39%	17.11%
Plaque	0%	69.47%	16.84%	13.68%
CCD status				
Negative	16.56%	45.86%	21.66%	15.92%
Positive	10.33%	54.96%	18.18%	16.53%

Table 2. Distribution of astrocytic morphological clusters across plaque domains and cognitive status. Astrocytes were classified into four morphological clusters based on principal component analysis and k-means clustering. The relative distribution (%) of each cluster is shown for non-plaque and plaque-associated domains, as well as for dogs with negative (CCD–) and positive (CCD+) cognitive status. A significant shift in cluster distribution was observed between plaque and non-plaque regions, with plaque-associated astrocytes showing enrichment of specific morphological phenotypes. Chi-square analysis confirmed a significant association between cluster distribution and domain ($\chi^2 = 24.871$, $df = 3$, $p = 1.643 \times 10^{-5}$). In contrast, no significant association was observed between cluster distribution and cognitive status ($\chi^2 = 5.035$, $df = 3$, $p = 0.1692$).

Glial cells coverage of A β plaques is not associated with cognitive status

Next, we applied classifiers protocols to assess glial cells coverage in A β plaque areas. Both microglia and astrocyte coverage of plaque area did not significantly differ

between cognitively impaired and intact dogs (Figure 8). This suggests that within plaque microenvironment, glial cells coverage doesn't significantly differ based on cognitive status. Despite the absence of statistical significance, microglial cells coverage was trending to increase in CCD- dogs which could be due to the morphological branching ramified microglial in cognitively normal dogs. Although astrocytes coverage of amyloid plaques did not show statistical significance, CCD+ dogs showed a higher trend in coverage.

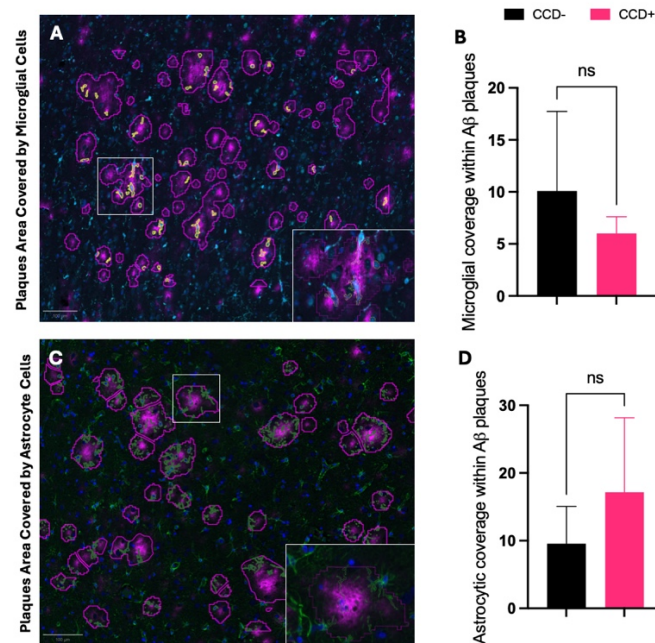


Figure 8. Glial coverage of amyloid plaques in canine cognitive dysfunction.

(A) Representative image of amyloid- β (A β) plaques (magenta) with microglial coverage (yellow signal within plaques), illustrating quantification of microglial area within plaque boundaries. Insets show higher magnification of plaque-associated microglia.

(B) Quantification of microglial coverage within A β plaques in dogs with negative (CCD-) and positive (CCD+) cognitive status. No significant difference was observed

between groups (ns). (C) Representative image of A β plaques (magenta) with astrocytic coverage (green signal within plaques), demonstrating astrocyte area occupancy within plaque regions. Insets highlight astrocyte–plaque interactions at higher magnification. (D) Quantification of astrocytic coverage within A β plaques in CCD– and CCD+ dogs. No significant difference was observed between groups (ns). Data are presented as mean $\pm$ SD.

Astrocytes show increased spatial association with A β plaques in dogs with cognitive dysfunction

To further evaluate plaque-centered glial responses, spatial analysis was performed to quantify microglial and astrocytic localization relative to the nearest A β plaque (Figure 9). For microglia, the number of cells detected at the 50 μ m threshold, 10 μ m threshold, and within plaque boundaries did not differ significantly between CCD+ and CCD– dogs (Figure 9, panels B-D). However, there were trending increases in CCD+ dogs compared to CCD– but the difference was not statistically significant.

In contrast, astrocytes showed significantly greater spatial association with plaques in cognitively impaired dogs. CCD+ dogs showed significantly higher astrocytes at the 50 μ m threshold, within 10 μ m of the nearest plaque, and significantly more astrocytes located within plaque boundaries compared to CCD– dogs (Figure 9, panels F-H). Together, these results indicate that astrocytic plaque association is increased in dogs with cognitive dysfunction, whereas microglial plaque proximity is unchanged.

Microglia and astrocytes exhibit substantial morphological heterogeneity in the aged canine brain, as demonstrated by multivariate analysis and unsupervised clustering. However, the proportional representation of microglial morphological clusters was not significantly associated with plaque domain or cognitive status. In plaque-centered analyses, overall plaque coverage by microglia and astrocytes was not significantly different cognitive status. In contrast, spatial analysis showed that astrocytes, but not microglia, were more strongly associated with A β plaques in cognitively impaired dogs. These findings support that astrocytic responses may be more tightly linked to the extracellular plaque microenvironment in CCD.

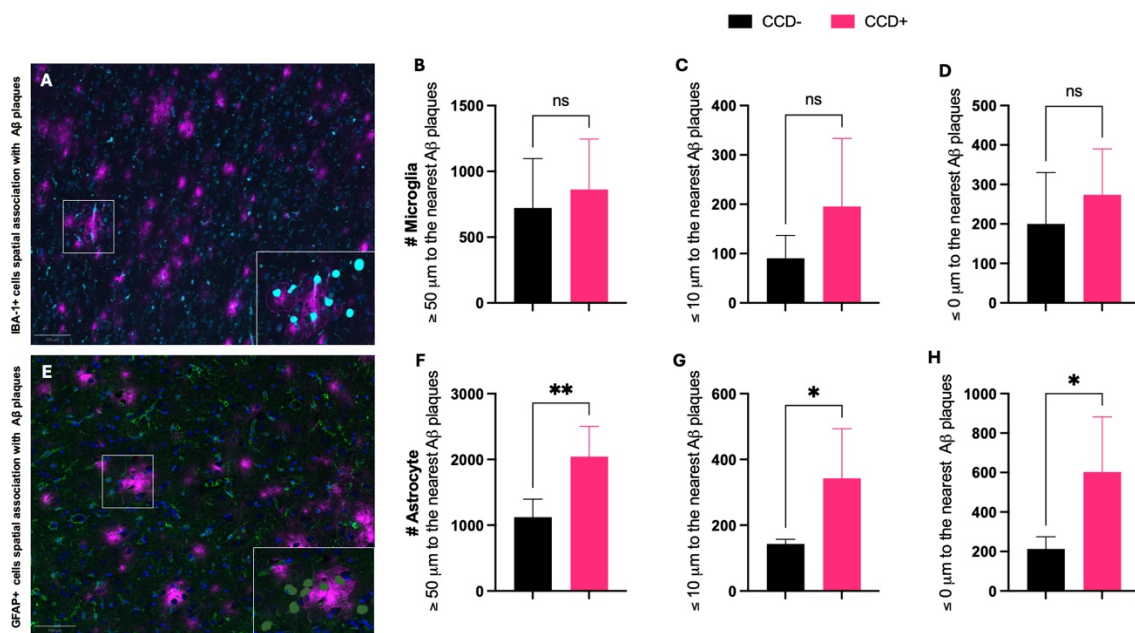


Figure 9. Spatial distribution of glial cells relative to amyloid plaques in canine cognitive dysfunction. (A) Representative image of Iba-1–positive microglia (cyan) in relation to amyloid- β (A β) plaques (magenta), illustrating spatial proximity analysis. Insets show higher magnification of microglia located near plaques. (B–D) Quantification of microglial cell counts at increasing proximity thresholds to the nearest A β plaque (≥ 50

μm , $\leq 10 \mu\text{m}$, and $\leq 0 \mu\text{m}$ [within plaques]) in dogs with negative (CCD-) and positive (CCD+) cognitive status. No significant differences were observed between groups at any distance threshold (ns). (E) Representative image of GFAP-positive astrocytes (green) in relation to A β plaques (magenta), with insets highlighting astrocyte-plaque interactions. (F-H) Quantification of astrocyte counts at increasing proximity thresholds to the nearest A β plaque ($\geq 50 \mu\text{m}$, $\leq 10 \mu\text{m}$, and $\leq 0 \mu\text{m}$ [within plaques]). Astrocyte numbers were significantly increased in CCD+ dogs compared to CCD- dogs across all distance thresholds (F: $**p < 0.01$; G-H: $*p < 0.05$). Data are presented as mean $\pm$ SD.

Rapamycin, Trazadone, and Cannabidiol potential efficacy in a naturally aging model

In this study, we leveraged the canine model as a translational bridge to evaluate whether available treatments could reduce A β pathological burden associated with aging and cognitive dysfunction in dogs. In a small cohort, aged dogs (>8 years old) with cognitive decline, as measured by CADES assessment, were randomized to receive placebo, trazodone, rapamycin, or CBD.

We first assessed A β pathological burden across dogs that received placebo, rapamycin, trazodone, or CBD (Figure 10 and table 3). A β plaque coverage varied across treatment groups compared with placebo; however, overall, CBD-treated dogs showed the lowest A β plaque burden. Glial cell counts were also informative, with the highest microglial and astrocytic counts observed in placebo-treated dogs compared with the other treatment groups (Figure 11-12 and table 3). Although these analyses were underpowered due to small sample size, they provide further insight into the use of a naturally aging canine model to evaluate repurposed treatments for cognitive dysfunction and AD-related pathology.

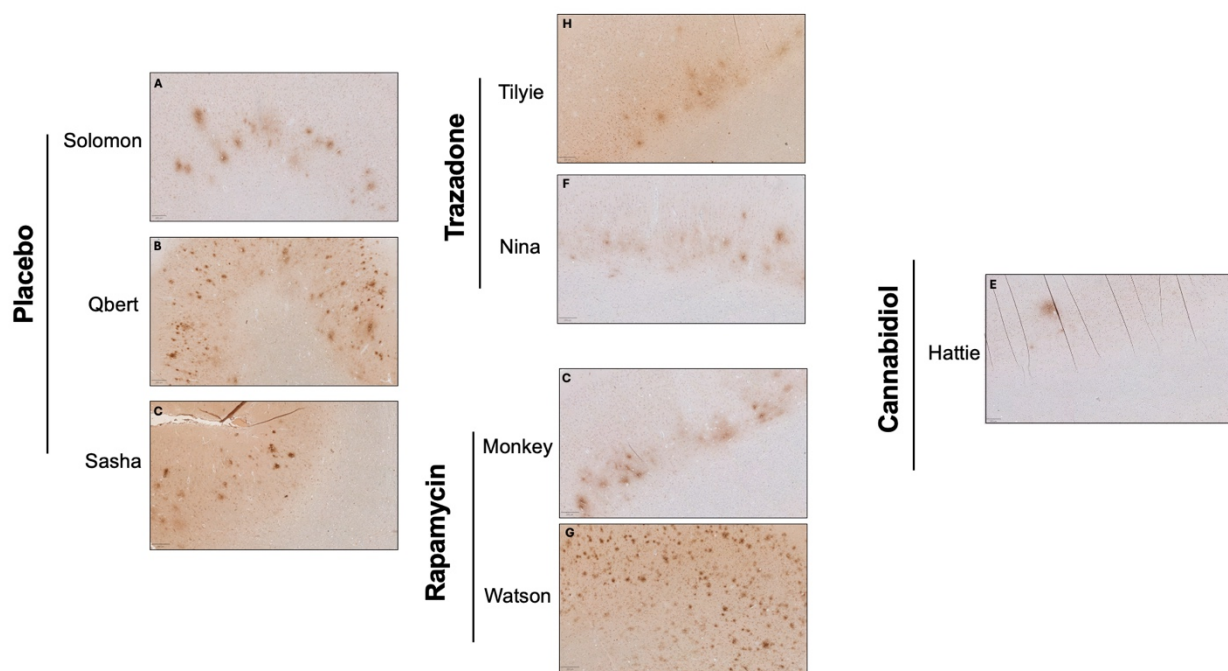


Figure 10. Representative images of Aβ pathological burden across treatment groups in aged dogs with cognitive dysfunction.

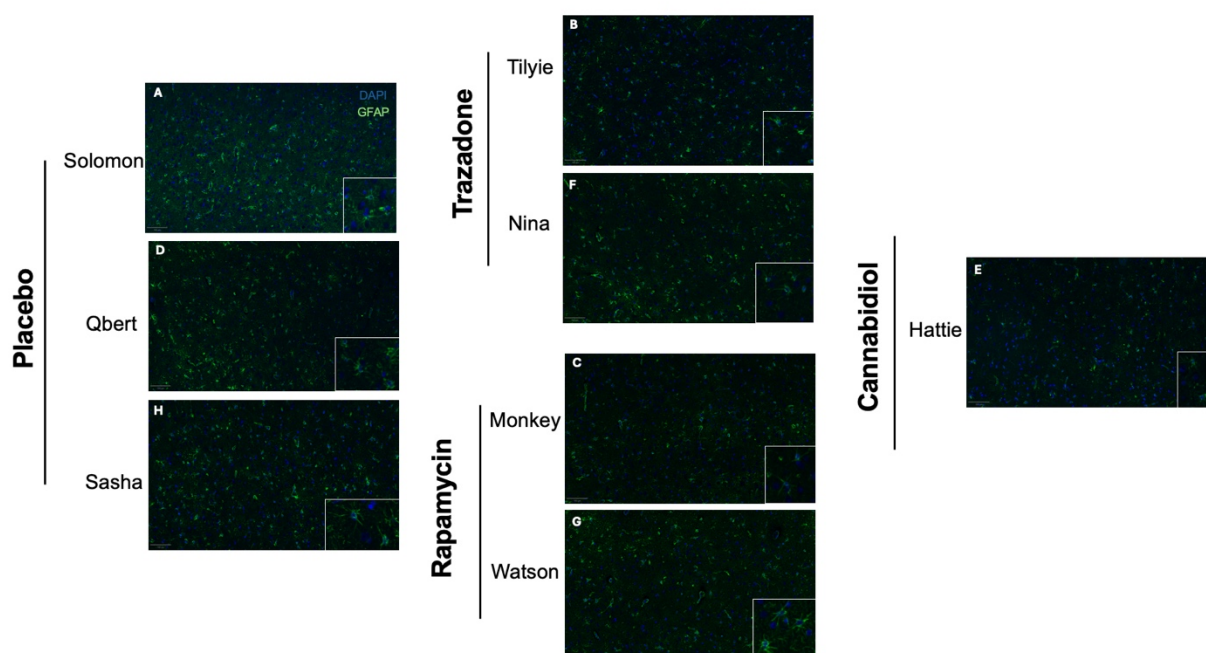


Figure 11. Representative images of astrocytes across treatment groups in aged dogs with cognitive dysfunction.

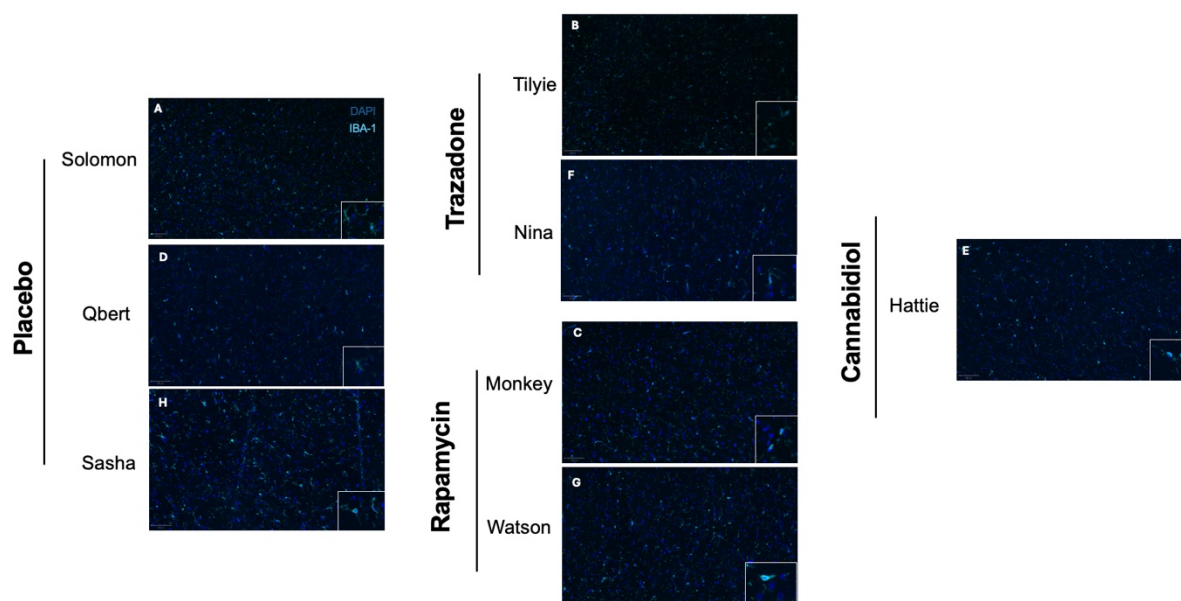


Figure 12. Representative of microglia across treatment groups in aged dogs with cognitive dysfunction.

ID	6E10 coverage	GFAP+/mm ²	IBA-1+/mm ²
Solomon (Placebo)	11%	603	144.0200209
Qbert (Placebo)	51%	334	74.8651734
Sasha (Placebo)	24%	322	254.330754
Tilyie (Trazadone)	13%	348	182.3993522
Nina (Trazadone)	22%	295	124.1465112
Monkey (Rapamycin)	12%	299	133.8663096
Watson (Rapamycin)	52%	282	113.485082
Hattie (Cannabidiol)	4%	319	124.1563551

Table 3. Quantification of A β plaque coverage and glial cells density across treatment groups in aged dogs with cognitive dysfunction. A β plaque coverage was variable across treatment groups, with the highest 6E10 coverage observed in Qbert (placebo; 51%) and Watson (rapamycin; 52%), while the lowest coverage was observed in Hattie (cannabidiol; 4%). GFAP-positive astrocyte density was highest in Solomon (placebo; 603 cells/mm²), whereas IBA-1-positive microglial density was highest in Sasha (placebo; 254.33 cells/mm²).

5.5 Discussion

Alzheimer's disease (AD) is a complex neuropathological disease that present with several modalities, including the presence of misfolded proteins and neuroinflammation mediated by glial cells (Kamatham et al., 2024). With the limited available naturally aging models, canines provide a translational relevance compared to other transgenic models (Sándor & Kubinyi, 2019). Dogs develop neurodegenerative disorder similar to AD, canine cognitive dysfunction syndrome (CCD). Our group and others have previously shown that aged canines develop neuropathological features similar to humans, including the accumulation of A β aggregates. Also, cognitively impaired canines typically present with higher neuroinflammation, including both microglial and astrocytic inflammatory responses. In this study, we show that aged dogs exhibit glial and A β -associated changes that parallel important features of AD, further enforcing CCD as a naturally occurring model of age-related neurodegeneration. By integrating glial cells morphological analysis and plaque focused spatial analysis, this study provides a broader view of how astrocytes and microglia respond to A β pathology in the aging canine brain. Overall, our findings indicate that glial remodeling in aged dogs is influenced by both cognitive status and the local plaque microenvironment and these relationships differ by cell type. Microglial morphological changes were more strongly associated with cognitive status (Figure 2), whereas astrocytic appeared to be more closely linked to plaque-associated pathology and spatial proximity to plaques (Figure 5). Together, these findings reinforce the concept that the inflammatory response of the aging canine brain is heterogeneous and cell-type specific, rather than reflecting a single uniform reactive state.

Microglia from cognitively impaired dogs adopted a less ramified and less structurally complex morphology (Figure 6, panel C). CCD+ microglial cells showed reductions across multiple branching-related parameters, together with reduced size-associated features and greater irregularity in shape. This overall pattern is consistent with microglial remodeling toward a more reactive state. Under normal physiological conditions, highly branched microglia are thought to mirror a global surveillant phenotype that continuously surveys the brain, whereas disease-associated microglia often undergo process retraction, simplification of branching architecture, and altered soma-to-process relationships (Eyo & Wu, 2019; Vidal-Itriago et al., 2022). In this study, the reduced branching complexity observed in CCD+ dogs may indicate a shift away from homeostatic surveillance and toward a more activated inflammatory phenotype. Importantly, these differences were observed when comparing dogs by cognitive status rather than plaque domain, suggesting that microglial morphological changes in this cohort may reflect broader cognition-associated changes across the tissue rather than being restricted only to extracellular plaque microenvironment.

This finding was unexpected given the widely recognized role of microglia in plaque sensing, clustering, and attempted phagocytic containment in AD. However, several factors may explain this pattern. First, microglial activation in aged brains may already be overwhelmingly widespread, such that plaque-associated and non-plaque regions both contain cells in a partially activated state (Islam et al., 2024; Norden & Godbout, 2013). Second, the non-plaque domain in aged dogs does not represent a truly inactivated baseline, as these areas still exist within an aging and pathologically vulnerable brain. Third, the impact of plaque proximity may be more strongly reflected in

functional state rather than structural morphology measurements (Godeanu & Cătălin, 2025).

In contrast to microglia, astrocytes showed more selective changes by cognitive status and even more pronounced modulations in relation to plaque domain. Astrocytes from CCD+ dogs showed reductions in several branching features, while most overall size and compactness variables remained unchanged (Figure 4). This suggests that astrocytic responses in cognitive impairment may involve subtle reorganization of branching structure without significant changes in cell size. This pattern may reflect early or selective reactive remodeling rather than a fully generalized hypertrophic response. Astrocytes are functionally diverse and can respond to disease through multiple pathways, including inflammatory signaling, metabolic support failure, altered neurotransmitter homeostasis, and changes in interactions with synapses and blood vessels (Allen & Eroglu, 2017; R. T. Han et al., 2021; Valles et al., 2023). Therefore, the relatively selective morphological changes observed in astrocytes in this study could indicate that their involvement in CCD is present but not fully captured in a simple reactive and nonreactive framework. On the other hand, plaque-associated astrocytes exhibited marked structural remodeling, supporting the concept that the extracellular A β environment is a major driver of astrocytic reactive phenotype (Figure 5).

The identified clusters emphasized that glial morphology in the aged canine brain can't be sufficiently described by a single variable alone. Both microglia and astrocytes exhibited substantial heterogeneity when analyzed through PCA followed by k-means clustering, indicating the presence of morphological subtypes rather than a single linear spectrum. For microglia, six morphologically distinct clusters were identified, spanning

irregular, moderately ramified, territorially expanded, amoeboid-like, elongated, and highly ramified forms. For astrocytes, four morphological clusters were identified, including highly branched, expanded, compact, and elongated morphologies. This heterogeneity is biologically important because glial responses in neurodegeneration are increasingly recognized as multidimensional, region specific and context dependent. Distinct morphologies may reflect differences in activation state, local environment, plaque interaction, or disease stage (García-Domínguez, 2026). However, the lack of significant differences in microglial cluster representation by plaque domain or CCD status suggests that while diverse morphologies exist, they may not separate into interpretable disease groups in this cohort.

A β Plaque coverage by microglia and astrocytes did not differ significantly between cognitively impaired and intact dogs (Figure 8). This suggests that the total proportion of plaque area occupied by glial signal may not be the most sensitive feature distinguishing cognitive groups. The trend towards an increase of microglial coverage in cognitively intact dogs raises the possibility that more ramified microglia cells in cognitively intact dogs have greater process coverage over plaque regions, which is supported by morphological more branched cells in CCD- dogs.

In contrast, the spatial analysis revealed a clearer disease-associated astrocytic pattern. Astrocytes in CCD+ dogs were significantly more abundant within 50 μ m or higher and 10 μ m of plaques distance to the nearest plaque (Figure 9). Also, within plaque boundaries, astrocytes were significantly higher in cognitively impaired dogs. These findings indicate that astrocytic recruitment or infiltration around and within

plaques is increased in cognitively impaired dogs, suggesting that they are more tightly linked than microglia to A β plaque microenvironment.

Several limitations should be considered when interpreting this study. First, the morphology analyses relied on selected representative cells, and although care was taken to standardize image preparation and cell inclusion, this approach may not fully capture the entire spectrum of glial morphology present within the pre-frontal cortex section. Second, morphology alone cannot define glial functional state, and the same general structural pattern may reflect multiple molecular phenotypes. Future studies integrating morphology with transcriptomic approaches would strengthen interpretation of these structural states. In addition, the treatment cohort evaluating rapamycin, CBD, and trazodone was relatively small, limiting power to detect potentially meaningful intervention-related effects. Finally, owner-based cognitive assessments such as CADES are highly valuable clinically, but pathological dissociation remains possible.

Despite these limitations, the present study provides evidence that glial remodeling in aged dogs is both biologically meaningful and context dependent. Microglia in cognitively impaired dogs exhibited reduced branching complexity and altered shape consistent with widespread disease-associated remodeling, while astrocytes showed particularly strong structural and spatial responses in plaque-associated environments. Although overall plaque coverage did not differ by cognitive group, spatial analyses identified increased astrocytic recruitment towards A β plaques in CCD+ dogs. Together, these findings support the idea that glial responses in the canine brain are shaped by both global cognitive decline and the local amyloid microenvironment. More broadly, they further establish aged companion dogs as a

relevant model for investigating the neuroinflammatory and plaque-associated changes that contribute to Alzheimer-like brain aging.

Conclusion

The findings in this study provide broader translational relevance. A major challenge in AD research is the limited ability of conventional transgenic models to recapitulate the spontaneous, aged, and heterogeneous nature of human disease. Companion dogs develop A β pathology, tau-associated changes, neuroinflammation, and cognitive decline in a naturally occurring context, making them uniquely valuable for translational investigation. The present results strengthen that relevance by showing that aged dogs not only develop AD-like lesions but show plaque-associated and disease-associated glial remodeling at the cellular level. This is important because glial cells are increasingly recognized as central pathological components related to disease progression and as potential therapeutic targets.

Chapter Six: Discussion

6.1 Overview of the dissertation and central findings

The overarching goal of this dissertation was to characterize canine cognitive dysfunction (CCD) using a multimodal framework that integrates neuropathology, neuroinflammation, glial morphology, plaque-associated glial responses, and fluid biomarkers relevant to Alzheimer's disease (AD). Across the studies presented here, several consistent themes emerged. First, aged dogs develop key pathological features that parallel important components of AD, including amyloid- β (A β) deposition, regionally distinct tau-related pathology, neuroinflammatory changes, and measurable cognitive decline. Second, these disease-associated changes are biologically meaningful, but they do not occur in a uniform manner across dogs, indicating substantial clinical and pathological heterogeneity. Third, glial responses are not simply generalized background features of aging, but they reflect both progressive disease status and the toxic A β plaque microenvironment at cellular level. Finally, biomarker and pathology findings together support the aged companion dog as a translationally relevant model for investigating early and intermediate AD-like disease in a naturally aging model.

A major strength of this dissertation is that it moved beyond a singular readout of pathological progression. Rather than relying only on behavioral changes assessments such as owner-assessment cognitive scores, only on pathology, or only on fluid biomarkers, this work examined how these domains intersect and influence pathological

changes. In neurodegenerative diseases, cognitive impairment reflects the cumulative effects of several converging pathways rather than a single lesion phenotype (Hein et al., 2025). The integration of plasma and CSF biomarkers, human AD neuropathological frameworks, quantitative pathology, and plaque-associated glial analyses allowed the dissertation to address not only whether aged dogs develop AD-like pathology, but also how these changes relate to one another. Collectively, the findings support the view that CCD is not merely nonspecific aging, but instead represents a complex neurodegenerative condition with meaningful overlap with human AD.

6.2 The aged canine brain recapitulates key features of Alzheimer's disease

One of the important conclusions of this dissertation is that the aged canine brain recapitulates multiple core features of AD, particularly those associated with early and intermediate stages of disease. Across the neuropathology-focused chapters, dogs showed substantial A β deposition, regionally restricted phospho-tau pathology, cerebral amyloid angiopathy in a subset of cases, and neuroinflammatory alterations involving both astrocytes and microglia. Importantly, these pathological lesions developed spontaneously in the aging companion dogs. This is a key translational advantage because it allows investigation of disease processes within the natural biological context of aging, environmental exposures, lifestyle variability, and genetic diversity.

The second and third chapters represented the neuropathological investigations of CCD and its' similarities to AD. The second chapter in particular was more associated with CCD characterization using human AD frameworks, including Thal-based amyloid distribution, Braak-based regional tau evaluation, and CERAD neuritic plaque

assessment. The results showed that most dogs demonstrated low AD neuropathologic change, with a smaller subset of aged dogs developing intermediate ADNC, while none met criteria for high ADNC. This pattern suggests that aged dogs most closely model the earlier stages of AD-related pathological progression, where amyloid deposition is evident, tau pathology is emerging but still limited, and mature neuritic plaques remain absent. Rather than weakening the model, this actually strengthens its translational value for addressing one of the most important unmet needs in AD research, which is to understand the earliest disease stages before advanced, end-stage pathology overwhelmingly dominates the brain.

The absence of neuritic plaques in the canine cohort is noteworthy. In human AD, neuritic plaques are a key feature of advanced ADNC and reflect plaque-associated dystrophic neurite injury. In the present work, widespread A β deposition occurred without the presence of classical neuritic plaque morphology that is usually seen in terminal AD patients. This finding suggests that aged dogs may model an earlier plaque state or a species-specific form of A β plaque biology in which diffuse amyloid deposition develops without progressing fully to neurotoxic neuritic plaques. This distinction is important because it indicates that canines should not be viewed as an end-stage AD model, but rather as a biologically relevant model for investigating early neuropathological changes, including A β accumulation, early tau involvement, and inflammatory responses prior to development of terminal end stage pathological lesions.

6.3 Clinicopathological relationships in canine cognitive dysfunction

This dissertation provides further insights on the association between cognitive impairment and pathological changes. The association between the two are linked but not perfectly aligned. In the biomarker chapter, measures such as NfL and A β _{42/40} ratio were associated with age and owner reported cognitive scores. In the neuropathology chapter, cognitively impaired dogs showed higher A β burden, specifically in cortical regions. On the other hand, tau pathology and other features were more variable. In chapter three, the amyloid-inflammatory dimension was independently associated with both age and cognitive impairment. Dogs that were more associated with tau-dominant axis weren't independently associated with age or cognitive scores, which highlights the fact that tauopathies or tau phenotypes in canines are not fully developed. Together, these findings show that CCD is biologically related to underlying neuropathology, and that cognitive decline cannot be minimized to a single pathological marker alone.

This imperfect alignment should not be interpreted as a weakness of the canine model. On the contrary, clinicopathological discordance is a well-recognized feature of human aging and dementia (Negash et al., 2013). In humans, substantial A β burden can occur in cognitively normal individuals, whereas cognitive impairment may at times reflect mixed pathology, synaptic dysfunction, inflammatory state, vascular contributions, or disease stage rather than A β or tau burden alone (Sarhan et al., 2025). A similar principle likely applies in aged dogs as well. Behavioral changes assessment tools, such as CADES and CCDR, are highly valuable and clinically relevant, but they capture the owner assessment of their pet behavior rather than the underlying pathological burden. Furthermore, postmortem pathology provides a limited view of tissue at end-

stage of the disease rather than the full temporal evolution of decline. The partial misalignment between clinical status and pathology in this dissertation therefore likely reflects true biological complexity, including resilience, regional vulnerability, timing of lesion development, and the contribution of multiple interacting mechanisms.

This dissociation between behavioral-cognitive changes and pathological progression may also be explained by phenomena well recognized in human Alzheimer's disease (AD) research, pathological resilience and pathological resistance (Latimer et al., 2019b). Pathological resilience refers to the preservation of cognitive function despite substantial neuropathological burden, whereas pathological resistance refers to the relative absence or limited accumulation of pathology despite advanced age (Bocancea et al., 2021; Dominguez et al., 2024). These concepts suggest that cognitive decline does not always align directly with neuropathological changes. Instead, the clinical expression of disease may depend on additional biological factors that influence whether pathological lesions translate into neuronal dysfunction and measurable behavioral impairment. Several mechanisms have been proposed to explain these clinicopathological discrepancies, including differences in cognitive reserve, synaptic integrity, neuroinflammatory responses, vascular health, and regional vulnerability of specific neural circuits.

This framework is particularly relevant to the present study because a subset of dogs showed discordance between cognitive status and neuropathological severity. For example, some dogs may develop substantial A β or tau pathology without marked behavioral impairment, suggesting a resilience-like phenotype. Conversely, other aged dogs may also show limited pathological accumulation despite advanced age, which

reflects a resistant-like phenotype. These patterns indicate that CCD, similar to human AD, cannot be determined solely by the presence or absence of hallmark lesions. Furthermore, the patterns emphasize that cognitive impairment may emerge when pathological burden exceeds the compensatory capacity of the brain or when pathology affects regions and cellular networks that are especially important for learning, memory, and behavior. This dissertation supports the need for integrative models in which cognition is interpreted alongside plaque burden, tau pathology, biomarker profiles, neuroinflammatory readouts, and broader multivariate patterns. Such an approach may ultimately prove more informative than the classical binary case-control classification alone.

6.4 Amyloid- β pathology emerges as a central and consistent feature

Among all findings presented in this dissertation, A β pathology was the most consistent disease-associated feature. In the biomarker chapter, the A $\beta_{42/40}$ ratio was associated with aging and cognitive status, especially in CSF, supporting its value as an indicator of underlying amyloid burden. In chapter three, all aged dogs showed neocortical A β deposition, with additional involvement of hippocampus, striatum, and occasionally midbrain, broadly paralleling hierarchical amyloid progression. In the neuropathological heterogeneity chapter, CCD+ dogs demonstrated significantly greater A β burden in both frontal cortex and hippocampus. These results position A β accumulation as a central pathological domain in the aging diseased canine brain.

This consistency is particularly important because it supports the concept that amyloid pathology in dogs is not incidental. Rather, it appears to be tied to both age and

cognitive decline, while also serving as a structural scaffold around which glial responses are organized. At the same time, the dissertation also emphasizes that A β alone is not sufficient to explain all biological variation. Some dogs with substantial amyloid burden may show less severe cognitive impairment, while other pathological processes, including tau-related changes and neuroinflammation, modify the overall disease processes. This is highly consistent with the current understanding of AD, where amyloid is considered an early and important driver, but not the sole determinant of clinical outcome. In this sense, the aged dog provides a useful early-intermediate model for investigating amyloid-centered disease processes in the context of natural aging without the artificial changes imposed by transgenic overexpressing models.

Another important point is that amyloid pathology in dogs appears to be regionally informative. The prefrontal cortex emerged as a particularly relevant site in several analyses, showing substantial plaque burden and disease-related differences. The hippocampus also demonstrated important pathological involvement, especially when considered alongside tau-related changes. This regional pattern reinforces the concept that frontal and limbic structures may be especially vulnerable in CCD and that these anatomical regions should remain central targets in future mechanistic studies. Other structures that also provided pathological insights include visual cortex and the striatum. Both regions showed an increasing involvement of amyloid pathology as canines age. However, the accumulation of amyloid pathology in these regions is relatively lower compared to the prefrontal cortex and hippocampus.

6.5 Tau-related pathology strengthens the Alzheimer-like nature of the canine model

Although tau pathology was less pervasive compared to amyloid across the dissertation, its presence is highly significant conceptually. Historically, one of the main criticisms of the canine model has been that dogs show A β deposition more readily than robust neurofibrillary tangles. The present dissertation adds to the growing evidence that this view is incomplete. Across chapters three and four, phospho-tau immunoreactivity was detected in a regionally restricted but meaningful pattern, particularly in the hippocampus and in selected cortical regions. The multivariate analysis in chapter four showed that tau exists along distinct pathological axes rather than an association with amyloid.

This is important because tau-related pathology is more closely associated with neuronal dysfunction and clinical progression in human AD. In several studies and through different testing modalities, tau shows a prognostic potential in association with degree of severity and progression. However, in CCD, tau doesn't follow a similar pathological progression due to several factors. Even if tau burden in dogs is not as extensive or anatomically advanced as in severe AD patients, its presence shifts the canine model beyond a simple "amyloid aging" framework. Instead, it supports the interpretation that aged dogs can develop multiple major protein aggregates that resembles AD. The observation that some tau patterns followed expected hippocampus to neocortex progression, while others were atypical, further emphasizes that canine tau pathology is both biologically relevant and heterogeneous. This heterogeneity may reflect disease stage, species-specific progression, epitope sensitivity, or the

coexistence of multiple tau-related processes that do not map perfectly onto human staging systems.

Although CCD shares several neuropathological features with AD, differences in lifespan and end-of-life trajectories between dogs and humans likely contribute to key discrepancies, particularly in the distribution and extent of tau pathology. Companion dogs are frequently euthanized at the request of their owners, often due to declining health, reduced quality of life, or increasing caregiving burden. As a result, relatively few dogs are allowed to progress to advanced stages of disease or die from natural causes.

In human AD, tau accumulation and neurofibrillary tangle formation become increasingly prominent at later stages of disease progression and are more closely associated with neuronal dysfunction and clinical decline. In contrast, the early termination of life in dogs may limit the opportunity for tau pathology to fully develop to the extent observed in advanced AD. Consequently, the comparatively modest or regionally restricted tau pathology observed in CCD should not be interpreted as a lack of disease relevance, but rather as a reflection of differences in disease duration and progression.

These considerations suggest that tau pathology in CCD has not yet reached its most advanced forms. This distinction is important for positioning the canine model within the broader context of AD research and highlights its particular value for studying early disease mechanisms rather than late-stage neurodegeneration.

It is also notable that tau did not consistently distinguish dogs based on cognitive status across all analyses. This may indicate that tau-related changes in dogs are more

variable, emerge later in the disease course, or is more regionally dependent than amyloid deposition. Alternatively, this pattern may reflect methodological limitations, including the assessment of a restricted set of tau epitopes or anatomical regions. Regardless, the presence of regionally localized tau pathology supports the AD-like nature of CCD, while the inconsistency of its clinical associations underscores the need for more refined approaches to measuring and staging tau in the canine brain rather than dismissing its relevance.

6.6 Neuroinflammation is not secondary background noise, but a central component of canine brain aging and cognitive decline

Another major conclusion of this dissertation is that neuroinflammation is not simply an accompanying feature of protein aggregation, but one of the major pathological drivers organizing disease expression in aged dogs. This was evident in several ways. In the biomarker chapter, GFAP showed age-related associations, and the general biomarker panel supported the idea that neuronal injury and glial responses are detected in dogs. In the third chapter, plasma GFAP correlated positively with plaque burden, linking peripheral biomarker signals to central amyloid pathology. In the fourth chapter, astrocytic and microglial markers contributed strongly to the amyloid-inflammatory dimension that was independently associated with age and cognitive impairment. Together, these findings support the view that inflammatory biology is integrated into the pathological architecture of CCD rather than simply appearing downstream consequence of cellular damage.

The pathological heterogeneity identified in Chapter Four further emphasizes the importance of neuroinflammatory processes in CCD. Both A β and tau contributed to

distinct pathological axes and demonstrated clear separation, highlighting that disease progression is shaped by multiple interacting mechanisms rather than a single linear pathway. Importantly, both axes showed strong involvement of glial cells, reinforcing the central role of neuroinflammation in the underlying pathology. The amyloid-inflammatory axis was particularly associated with increased microglial and astrocytic responses, while tau-dominant axes were more aligned with astrocytic responses, indicating that astrocytes may play a prominent role in relation to tau-related pathology. This interpretation is especially important given the contemporary shift in AD research toward integrated models that place glia at the center of disease progression.

6.7 Astrocytes and microglia respond differently to disease status and the plaque microenvironment

One of the most informative findings in this dissertation is that astrocytes and microglia do not behave identically. Instead, each cell type appears to encode different aspects of disease biology. Microglial morphology was more strongly altered by cognitive status, whereas astrocytic changes were more strongly linked to plaque-associated microenvironments and spatial relationship to plaques. This divergence suggests that the two major glial populations may occupy distinct roles in the progression of CCD.

Microglia from cognitively impaired dogs displayed reduced branching complexity, fewer junctions and endpoints, smaller size-related features, and greater irregularity of shape. This overall pattern is consistent with structural remodeling away from a highly surveillant ramified state and toward a more reactive phenotype. Importantly, these changes were more strongly associated with CCD status than with

simple plaque proximity. This suggests that microglial remodeling in aged dogs may represent a broader tissue-level response to disease rather than a purely plaque-restricted phenomenon. Such a pattern is biologically plausible, as microglia are capable of responding to diffuse inflammatory signals, synaptic alterations, subtle neuronal injury, and soluble protein aggregates throughout the brain.

Astrocytes, by contrast, showed a different pattern. Although cognitively impaired dogs had selective reductions in branching features, the most dramatic astrocytic differences were seen in plaque-associated domains. Plaque-associated astrocytes were enlarged, showed reduced fine branching, and exhibited increased area, perimeter, span, and mean radius, indicating marked structural remodeling in the immediate amyloid microenvironment. In spatial analyses, astrocytes were also more strongly spatially closer to plaques in cognitively impaired dogs, including at 50 μm , 10 μm , and within plaque boundaries. These findings suggest that astrocytes may be especially sensitive to local amyloid-related aggregates and may play a major role in structuring the plaque-centered inflammatory environment in cognitively impaired dogs.

This dissociation between cell types is conceptually important. It argues against reducing neuroinflammation to a single generalized response. Instead, it suggests that microglia may reflect more diffuse disease-associated remodeling, whereas astrocytes may better capture localized plaque-centered inflammatory processes. Also, it is consistent with current thinking in human AD, where microglia are often viewed as rapid immune responders and astrocytes as crucial modulators of local tissue homeostasis, plaque-associated remodeling, metabolic support, and inflammatory signaling. The

canine model is therefore especially useful because it reveals these cell-type-specific patterns within a spontaneous disease setting.

6.8 Pathological heterogeneity is a defining feature of aged dogs with cognitive decline

A particularly strong contribution of this dissertation is the demonstration that pathological heterogeneity is not a minor detail but a defining feature of CCD. In the fourth chapter, principal component analysis and hierarchical clustering showed that aged dogs do not fall along a single simple disease continuum. Instead, multiple pathological patterns were identified, including low pathology, tau-enriched, and mixed amyloid-inflammatory patterns. The major dimension most strongly associated with age and cognition reflected an amyloid-inflammatory axis, whereas other dimensions captured more selective tau-related variation. This finding is highly informative because it indicates that CCD is not a uniform neuropathologic disease and that disease-associated variation is structured rather than random.

The findings in chapter four help explain why single measures do not always perfectly distinguish dogs based on cognitive status. If animals arrive at cognitive decline through partially different pathological routes, then no single biomarker or histologic measure should be expected to capture all clinically or pathological variation. Furthermore, pathological heterogeneity increases the translational relevance of the dog model. Human AD is itself heterogeneous, with variation in plaque burden, tau progression, inflammatory state, vascular involvement, and resilience (Duara & Barker, 2022b). A model that captures this variability may actually be more informative than a highly standardized but biologically simplified model. The identification of distinct

pathological patterns provides a foundation to more refined future studies, including subgroup analyses, targeted biomarker development, and mechanistically targeted therapeutic interventions. The presence of heterogeneity should not be viewed as noise that complicates analysis. Rather, it should be viewed as one of the most important biological signals in the dataset. It reflects the reality that neurodegenerative disease is a network of interacting processes, not a single linear lesion. The canine model is valuable precisely because it preserves that complexity and variation.

6.9 Biomarkers provide an important bridge between clinical status and brain pathology

The biomarker chapter showed that age and cognitive decline in dogs are reflected by changes in plasma and CSF markers relevant to AD, particularly NfL and $A\beta_{42/40}$ ratio. Plasma NfL emerged as one of the strongest candidates, showing positive relationships with age and cognitive impairment, while CSF and plasma NfL were also correlated. The $A\beta_{42/40}$ ratio, particularly in CSF, showed sensitivity to cognitive impairment and amyloid-related change. Also, the glial marker GFAP showed age-related relationships and in the third chapter. Plasma GFAP correlated with plaque burden, further enforcing the notion that biomarkers can capture complementary dimensions of disease, including axonal injury, amyloid biology, and glial activation.

The dissertation appropriately reveals that no single biomarker is sufficient on its own. NfL is promising but nonspecific, because it reflects neuronal injury broadly rather than AD-like pathology uniquely. GFAP appears biologically meaningful, but not uniformly robust across all analyses. $A\beta_{42/40}$ ratios may be more pathology-linked, but their signal differ between plasma and CSF. This reinforces the conclusion that a

biomarker panel, rather than a single marker, is likely to be most useful in CCD, which mirrors the current shift in approaches in human AD. The use of panels integrating amyloid, tau, neuronal injury, and inflammatory markers are increasingly favored. The canine findings therefore strengthen the translational bridge between veterinary and human biomarker research.

Another important contribution of this dissertation is that biomarker findings were not isolated from central pathology. By linking fluid measures of GFAP, NfL, and A β ratios to brain pathology, this dissertation moves beyond descriptive biomarker work and toward a clinicopathological framework. This is exactly the type of integrative approach needed if biomarkers are to become useful in both research and eventual veterinary practice.

6.10 Therapeutic implications and the promise of repurposed interventions

The final experimental direction in this dissertation extended into treatment-relevant territory by considering repurposed compounds such as rapamycin, cannabidiol, and trazodone in a smaller cohort. Although these analyses were exploratory and more limited in size, their inclusion is important. A major challenge in AD research is the repeated failure of interventions that were promising but did not translate effectively into spontaneous disease. A naturally aging canine model offers a valuable translatable path in which therapeutic candidates can be evaluated in natural AD model with complex disease processes, including mixed pathology, variable inflammatory responses, and heterogeneous clinicopathological presentations.

The therapeutic relevance of this dissertation therefore lies in the framework established. Through the identification of which disease domains are most strongly linked to cognitive decline, which biomarkers track closely with pathology, and which glial responses are most closely linked to pathological lesions, this work helps define what future interventions should target.

6.11 Strengths of the dissertation

Several strengths enhance the significance of this dissertation. One is the use of a naturally aging, translationally relevant model rather than a transgenic laboratory rodent model. Another is the integration of multiple complementary levels of analysis, including cognitive surveys, plasma and CSF biomarkers, standardized neuropathologic frameworks, quantitative histology, multivariate clustering, and plaque-centered glial analyses. The application of human AD criteria to canine tissue is another major strength because it creates a shared diagnostic structure between veterinary neuropathology and human neurodegenerative research. The inclusion of both conventional pathological parameters and multivariate analyses also strengthens the work by capturing both canonical disease hallmarks and the heterogeneity that lies beyond individual groups.

6.12 Limitations

Several limitations should also be acknowledged. First, many of the analyses are cross-sectional, which limits inference about temporal ordering. Although the dissertation strongly supports associations among pathology, biomarkers, and cognition, it cannot fully determine which changes precede others within individual animals.

Longitudinal work will be necessary to map disease trajectories more directly. Second, cognitive assessment relied primarily on owner-reported instruments. These tools are clinically valuable and well established, but they remain indirect measures of underlying neuropathology and may be influenced by owner perception or behavioral variability. Third, while the neuropathology analyses were substantial, they still sampled selected regions and markers rather than the full spectrum of possible mixed pathologies. Additional work on vascular changes, synaptic markers, oligomeric species, white matter injury, and other age-related lesions would further refine the canine disease framework. Fourth, morphology analyses relied on structural states rather functional transcriptomic phenotypes. Future work focused on integrating transcriptomic with morphological changes would be valuable. Finally, the treatment cohort were based on a smaller cohort, so they should be interpreted cautiously and viewed as exploratory rather than definitive.

6.13 Future directions

The findings in this dissertation point to several important future directions. One priority is longitudinal study design. Following dogs over time with repeated biomarker sampling, cognitive testing, and eventually postmortem neuropathology would provide a much more powerful framework for understanding disease progression. Such studies could determine whether biomarker changes precede clinical decline, whether inflammatory markers track worsening cognition, and whether specific pathological subtypes follow distinct trajectories.

A second priority is deeper characterization of tau pathological changes in dogs. The present work supports the relevance of tau in CCD but also shows that it is variable and regionally restricted. Future studies should examine more tau epitope, additional brain regions, and potentially soluble versus insoluble tau fractions to better determine how canine tau relates to human disease pathology. A third priority is more detailed analysis of the plaque microenvironment, including multiplex imaging or spatial transcriptomics to define the molecular identities of plaque-associated astrocytes and microglia. The morphology and spatial analyses in this dissertation provide a strong foundation for such work, but functional and transcriptomic are needed to identify the functional phenotypes underlying these morphological states.

Another major direction involves refining biomarker panels for veterinary and translational use. Plasma-based tools are especially attractive because they are minimally invasive and more feasible in clinical settings. The results here support the continued development of multi-analyte biomarker panels that combine injury, amyloid, and inflammatory measures rather than depending on a single marker. Finally, future therapeutic studies should take advantage of the pathological heterogeneity identified here. Rather than treating all aged dogs as a uniform disease group, intervention studies may benefit from stratifying animals by biomarker profile, cognitive severity, or pathological subtype. This may increase sensitivity to treatment effects and better reflect precision-medicine approach in human neurodegeneration.

6.14 Concluding remarks

In summary, this dissertation demonstrates that aged companion dogs develop a multidimensional pattern of neuropathology that includes amyloid deposition, regionally restricted tau-related changes, neuroinflammation, biomarker alterations, and cognitive decline. These features do not occur uniformly but instead reveal substantial clinicopathological and neuropathological heterogeneity. That heterogeneity is not a weakness of the model; rather, it is one of its greatest translational strengths. Across the chapters presented here, amyloid pathology emerged as a central and consistent feature, neuroinflammatory responses proved to be biologically meaningful and cell-type specific, and biomarker analyses showed promise for linking clinical status to underlying brain disease. Together, these findings support the aged companion dog as a valuable naturally occurring model of early and intermediate Alzheimer-like brain aging. More broadly, they show that understanding CCD requires an integrated framework in which proteinopathies, glial biology, biomarkers, and cognition are interpreted together rather than in isolation.

This dissertation carries significance beyond neuropathological changes in aged dogs. It contributes to a broader understanding of cognitive decline in aged dogs both as naturally occurring disease of companion animals and as a comparative model system for AD. Through the integration of multiple modalities, this work helps bridge the gap between clinical behavioral changes and the underlying biological processes occurring in the aging canine brain, providing researchers with a framework for studying CCD as a heterogeneous neurodegenerative condition rather than a single uniform disease process. For veterinarians, this work emphasizes that cognitive dysfunction in aged

dogs has a measurable biological basis that can be investigated through neuropathological characterization and antemortem fluid biomarkers, and that the clinical signs associated with CCD may reflect diverse and distinct underlying pathological processes. For dog owners, this dissertation reinforces that cognitive decline in aged dogs represents measurable brain changes rather than normal aging, encouraging earlier veterinary consultation and a better understanding of behavioral changes, while also highlighting the value of owner-based assessment tools as practical diagnostic instruments that contribute to improved diagnostics and more effective treatment strategies.

Together, these findings highlight the value of the aged dog in improving clinical recognition and care for CCD, while also supporting its role as a naturally occurring model for human neurodegenerative disease. By characterizing both shared and distinct features of CCD and AD, this dissertation helps bridge veterinary and human medicine through a bidirectional approach in which discoveries in companion animals can improve animal health while also providing translational insight into the mechanisms of human aging and dementia

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