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A sequential deposition of amyloid beta oligomers, plaques and phosphorylated tau occurs throughout life in the canine retina

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Abstract

Aims: Cerebral amyloid burdens may be found in otherwise cognitively intact adults, often not showing worsening deficits with passing years. Alzheimer's transgenic rodents have been widely used to investigate this phenomenon, but a spontaneous disorder in other animals, such as dogs that cohabit with humans and thus may have some shared environmental risks, may contribute and offer opportunities not possible in the smaller laboratory animals. In animals, the spontaneous disorder most comparable to Alzheimer's disease (AD) affects mature to aged dogs and is designated canine cognitive dysfunction. Motivated by AD, many studies have revealed that amyloid progressively accumulates in the canine central nervous system, including the retina. Here, we investigated whether deposits of amyloid and/or tau can be found in the canine retina of neurologically normal animals from the first year of life to the elderly. Suppose canine ocular amyloid and tau are present from early life. In that case, that raises the question of whether similar patterns of accumulation occur in man, whether as part of aging, AD, or other.

Methods: This study used eye tissues from 30 dogs with a variety of ophthalmic or other orbital disorders, of which 7/30 were 1-2 years old. Tissues were subdivided into dogs of three different age groups: young (1-5 years old), middle (6-10 years old), and old (≥ 11 years old).

Results: Following immunostaining of tissue sections with nanobodies against retinal $A\beta_{1-40}$ and $A\beta_{1-42}$ oligomers, and antibodies against Aβ plaques (Aβp) and hyperphosphorylated Tau (p-Tau), our investigations revealed that accumulation of $A\beta_{1-40}$ and $A\beta_{1-42}$ oligomers were widespread in the retina in all age groups. In contrast, Aβp were detected in the middle and old age groups but not in the young age group. Furthermore, p-Tau staining was observed in four old dogs only, while other dogs were p-Tau free. Interestingly, both Aβo and Aβp co-localized in the middle and old age groups of dogs. Moreover, diffuse granular p-Tau co-localized with intracellular Aβo in the old age group. Finally, we also observed co-localization of Aβo and Aβp in the retinal vasculature which might be similar to cerebral amyloid angiopathy associated with AD.

Conclusion: As far as we know, the presence of amyloid and tau in the canine retina is hitherto unreported. If similar, early-in-life subclinical retinal deposits occur in a human cohort perhaps identified by AD genetic risk factors, following this group may offer the prospect of preclinical therapeutic intervention in imminent dementia, a strategy recognized as likely necessary to impact this burgeoning disorder.

Keywords: Alzheimer's disease, canine cognitive dysfunction, retina, early diagnosis, Aβo, Aβp, p-Tau

INTRODUCTION

The principal neuropathological lesions in Alzheimer's disease (AD) brains include extracellular neuritic and/or diffuse plaques containing amyloid-beta (Aβ), intracellular tau protein (p-Tau) in the form of neurofibrillary tangles (NFTs) in addition to cerebral amyloid angiopathy (CAA), ubiquitin, severe synaptic loss, neuronal death, and brain atrophy[1-4]. While the deposition of Aβ in the human brain has traditionally been accepted as a major hallmark of AD[5], its accumulation is also observed in about 20% of cognitively unimpaired, aged individuals[6,7]. For decades, the association of Aβ with AD has been demonstrated, but the significance and impact of Aβ accumulation in healthy individuals are both poorly understood[8] and a source of confusion. For example, previous studies did not establish a clear correlation with memory loss in the aging brain[9-11]. Previous reports documented the progressive accumulation of both Aβ plaques (Aβp) in the brain[12,13] and Aβ oligomers (Aβo) in the brain and periphery of cognitively unimpaired individuals[14,15]. A study by Lesne *et al.* measured the levels of three Aβo "species", including Aβ trimers, Aβ*56 and Aβ dimers, in brain tissues from 75 cognitively unimpaired individuals, including young children and adolescents[14]. The authors showed that Aβ trimers were present in the central nervous system (CNS) of children and adolescents, and their levels increased progressively with age, suggesting that this particular Aβo could be used to track the potential progression into AD from a very young age. Another study investigated the relationship between amyloid levels and memory performance[15]. This study, which included 147 participants divided into three groups of adults 30-49, 50-69, and 70-89 years of age, established a clear relationship between episodic memory performance and amyloid accumulation in the youngest group[15]. A longitudinal study by Hanseeuw *et al.* demonstrated a correlation between Aβ/p-Tau and declining cognition in 60 clinically normal individuals aged between 65-85 years[16]. The authors concluded that there was a positive correlation between Aβ/p-Tau positron emission tomography (PET) and cognition, where participants with high Aβ and tau were at higher risk of developing mild cognitive impairment (MCI)[16]. These studies highlighted the importance of investigating and characterizing normal aging in cognitively unimpaired younger individuals to identify those at risk of progressive increase of AD-associated neuropathology changes and cognitive impairment and establish a time frame for early diagnostic and therapeutic intervention. However, such studies are difficult to implement in human subjects and will take decades to deliver any meaningful outcome[8,17].

Currently available transgenic animal models of AD do not replicate the subtle clinical and pathological features of the disease, as demonstrated by their lack of reproducible therapeutic outcomes[18,19]. As to the dog's age, there is an anatomically defined accumulation of Aβ peptides in the CNS, while less is known about cerebral p-Tau deposition. Some animals will develop a progressive cognitive decline with loss of learned behaviors and memory, a syndrome named canine cognitive dysfunction (CCD)[20-24], with CNS changes similar to the neuropathological hallmarks characteristic of AD[25-28]. In those destined to develop AD, amyloid precursor protein (APP) is sequentially cleaved by β and γ secretase leading to the production of Aβ peptide fragments (36-43 amino acids), which then aggregate and deposit as plaques[4,5,29]. The canine APP and Aβ peptides are 98% and 100% identical to their human counterparts[24]. In older dogs and others affected with CCD, Aβ deposits as diffuse plaques, while the dense core of humans' mature plaques is very rare or absent[20,21,27,30].

There are two major isoforms of Aβ, $Aβ_{1-40}$ (~80%-90%) and $Aβ_{1-42}$ (~5%-10%) and three major assemblies[31-34] recognized in man and animals/dogs, including monomeric Aβ, Aβo containing 12-24 monomers which become elongated to form protofibrils and finally insoluble fibrils[35]. Among these three stages, Aβo is thought to be the most toxic to neurons and responsible for the structural and functional pathologic changes associated with AD[36-39]. Likewise, a previous study reported that cognitive decline occurs before the accumulation of Aβp in CCD, supporting the premise that earlier assembly states of Aβ may be the toxic species[23]. In addition, CAA, ubiquitin, and severe synaptic loss have also been reported in CCD[20,40-43]. Another cardinal pathological hallmark of AD is the accumulation of p-Tau[3,4]. Several phosphorylation sites were identified on tau in aged dogs including Thr181[42], Ser422[43], Ser202/Thr205[40,43], Ser396[30,40,43], Ser189, and Ser207[44]; however, demonstrating the presence of NFTs has been infrequent compared to the consistent demonstration of amyloid oligomers and diffuse plaques. A study by Schmidt and colleagues using anti-pT205, AT8, AT100, PHF-1, and anti-pT422 antibodies seeking the presence of tau pathology in 24 dogs aged between two and nineteen years, showed that three 13-15-year-old dogs displayed p-Tau and only one 15-year-old Pekingese dog displayed NFT-like appearance[43].

In AD, visual disturbances are one of the early complaints and include loss of color vision, impairment of peripheral vision and object recognition, contrast sensitivity, and decreased visual memory and perception[45-47]. A recent longitudinal study of 1349 older adults showed that poor visual acuity paralleled the development of dementia[48], suggesting the possibility that ocular disturbances can be used as an early predictor of dementia risk in the older population[48]. Moreover, post-mortem studies of AD and animal models of AD demonstrated a strong association between retinal accumulation of Aβo[49,50]. Aβ plaques[51,52], p-Tau[53,54] and brain depositions and cognitive decline, where retinal Aβo was shown to deposit earlier than the brain and before deficits in cognition. Although, to our knowledge, age-dependent retinal deposition of Aβo and/or Aβp has not been investigated in AD and cognitively unimpaired individuals, including children and adolescents, our recent studies in AD mice models confirmed the conversion of cerebral and retinal Aβ o to Aβp in an age-dependent manner, where retinal Aβo was detected as early as 3-month old APP/PS1 mice, before brain pathology and cognitive decline were observed[49,55].

In this study, we investigated the retinal accumulation of Aβo, Aβp, and p-Tau in three age groups of genetically diverse and neurologically intact populations of dogs. Here, following immunohistochemistry and immunofluorescence analysis, we confirmed the presence of retinal $Aβ_{40}$ and $Aβ_{42}$ oligomers, Aβp, and p-Tau. Retinal $Aβ_{40}$ and $Aβ_{42}$ oligomers deposition was conspicuous and widespread and observed in all age groups, including the young 1-5-year-old neurologically intact group. Moreover, retinal co-localization of

$A\beta_{40}/A\beta_{42}$ oligomers and Aβp was observed in few middle-aged dogs and most dogs in the old neurologically intact age group, while retinal co-localization of $A\beta_{40}/A\beta_{42}$ oligomers and p-Tau was only seen in the old neurologically intact age group of dogs. Morphologically, extracellular Aβp deposits appeared as small, dot-like rounded deposits, while intracellular p-Tau deposits adopted a diffuse appearance in the retinal layers[54]. No NFTs or neuropil threads were observed in these dogs. Taken together, these results highlight the importance of further investigations of AD-related pathology in the retina of presymptomatic children and adolescents to gain insight into disease progression and potentially identify early at-risk individuals to help implement speedy therapeutic interventions.

METHODS

Dog eye samples and animal ethics

Sections of eyes used in this study were prepared from archived cases in the Comparative Ocular Pathology Laboratory of Wisconsin (COPLOW) at the Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison. Surgical enucleations had been submitted by practicing veterinarians to COPLOW for routine pathological diagnosis. These canine eyes came from 30 random, genetically diverse dogs (15 different breeds) ranging from 1 to 16 years of age with a broad variety of ocular disorders [Tables 1 and 2]. Such tissues, historically submitted for disease investigation purposes, are not subject to approval by institutional animal ethical regulations.

Tissue preparation

Enucleated eyes were submitted to the laboratory fixed in 10% neutral buffered formalin. On receipt, they were examined for gross abnormalities before trimming, followed by processing overnight using a Leica ASP300S tissue processor (Leica biosystem, Wetzlar, Germany). Tissues were then embedded in paraffin blocks and sectioned at 4μm thickness using a Leica RM2235 microtome (Leica biosystem, Wetzlar, Germany) and placed on charged slides. Sections were then stained with hematoxylin and eosin (H&E) and Congo red (CR). Further sections were used for immunohistochemistry (IHC) and immunofluorescence (IF) staining, and both upper and lower parts of the retina were assessed.

Hematoxylin and eosin staining

Paraffin sections were dewaxed by two changes of absolute xylene for 5 min each. Sections were rehydrated using two changes of 100% ethanol for 2 min each, 95% ethanol for 3 min, 70% ethanol for 2 min, and finally rinsed in deionized water for 2 min. H&E stain was then performed by adding Gill ll Haematoxylin solution (Leica Biosystems, Wetzlar, Germany), followed by 1% acid alcohol and subsequently eosin stain (Leica Biosystems, Wetzlar, Germany). Finally, the sections were dehydrated with increasing concentrations of ethanol, from 70%, 95% to 100%, then dewaxed by two changes of xylene and finally mounted with xylene-based mounting media (Sigma Aldrich, Missouri, United States)[56]. H&E staining was used to assess retinal morphology, the neuronal population within the eye.

Congo red staining

Initially, the CR working solution was prepared by mixing 50 ml Congo red solution and 0.5 ml potassium hydroxide solution supplied in the Congo red amyloid special stain kit (Leica Biosystems, Wetzlar, Germany). Retinal sections were placed in the working solution for 20 min and then rinsed in 5-8 changes of deionized water. This was followed by staining with Gill ll Haematoxylin (Leica Biosystems, Wetzlar, Germany) for 1-3 min and rinsing in 3 changes of deionized water. Sections were then dehydrated in two changes of 95% alcohol followed by three changes of absolute alcohol for one minute each. Finally, the sections were cleared in two changes of xylene and mounted in a xylene miscible medium. Amyloid fibrils appeared as dull to red brick under light microscopy (Olympus CX 43, Shinjuku, Tokyo, Japan) and apple green birefringent under polarized light (Olympus CX 43, Shinjuku, Tokyo, Japan).

Table 1. Signalment for 30 dogs and their retinal Aβ oligomers and plaques IHC scores

Age groups	Age (year)	Sex	Breed	Size	Pathological Aβ IHC findings in the dog retina	
					A11 - Aβ oligomers	4G8 - Aβ plaques
Young (1-5 years)	1	SF	Staffordshire terrier	Medium	+	-
	1.4	M	Siberian husky	Medium	-	-
	1.8	SF	Mixed breed	ND*	+++	-
	1.10	SF	Siberian husky	Medium	++	-
	2	F	Chihuahua mix	Toy	+++	-
	2	M	Bichon frise mix	Small	+	-
	2	NM	Shih Tzu	Small	+	-
	3	NM	German shepherd	Large	+++	-
	3	F	Giant schnauzer	Large	+	-
	3.6	M	Siberian husky	Medium	+	-
Middle age (6-10 years)	7	NM	Hound mixed	Medium	-	-
	8	SF	Bedlington terrier	Small	-	-
	8	SF	Cocker spaniel	Medium	-	-
	8	M	Mixed breed	ND*	-	-
	8	M	Cocker spaniel	Medium	+	-
	8	F	German shepherd mix	Large	-	-
	8	SF	Great dane dog	Giant	-	-
	9	M	Jack Russell terrier dog	Small	-	-
	9.5	SF	Boxer dog	Large	-	-
	9.9	SF	Cocker spaniel	Medium	-	-
Elderly (11-16 years)	11	NM	Mixed breed	ND*	-	-
	11	NM	Beagle	Small	-	-
	11.9	SF	Bouvier des flanders	Large	+	-
	12	SF	German shepherd	Large	-	++
	12	SF	German shepherd mix	Large	-	-
	12	NM	Husky mix	Medium	-	-
	12.8	NM	Shih-tzu	Small	-	++
	13	NM	Border collie	Medium	-	-
	15	SF	Basset hound	Medium	-	+
	16	SF	Shih Tzu	Small	-	-

Sizes were determined according to the American Kennel Club (AKC)[76]. Size range between 34 - ≥ 54 kg is considered "giant", 24-38 kg is considered "large", 15-29 kg is considered "medium", 3-15 kg is considered "small", and 0.9-4 kg is considered "toy". Aβ oligomers and Aβ plaques staining intensity were semi-quantitatively analyzed and scored across the retinal layers under the brightfield microscope (Olympus CX 43, Shinjuku, Tokyo, Japan). The total area was examined at 40 magnification and categorized into no immunostaining "-"; low immunoreactivity found only in limited areas of the retinal layers "+", moderate immunoreactivity where Aβ deposits were more apparent "++"; and finally strong immunoreactivity with widespread A11 and 4G8 positive Aβ labeling were exhibited "+++". ND*: Not determined; SF: spayed female; F: female; NM: neutered male; M: male; IHC: immunohistochemical.

Immunohistochemical staining of amyloid-beta plaques and amyloid-beta oligomers

Eye sections that contained the retina were pre-treated using the 2100 antigen retriever (Aptum Biologics Ltd, Southampton, United Kingdom) to expose the target epitopes. The sections were then treated with 90% formic acid for 5 min at room temperature (RT) followed by cell membrane permeabilization, achieved by using 0.1% Triton X for 1 min before the addition of 0.3% H_2O_2 for 15 min to inactivate endogenous peroxidases. Sections were then blocked with protein block serum-free (Agilent, City, Country) for 15 min. The sections were then stained for 1 h with the following primary antibodies in phosphate-buffered saline (PBS): mouse purified 4G8 anti-$A\beta_{17-24}$ (1:500; Bio legend, San Diego, CA, USA) or A11 rabbit anti-Aβo (1:250; Merck Millipore, Burlington, MA, USA) antibodies. After washing with PBS, sections were

Table 2. Signalment for 30 dogs, their ophthalmological disorders, and retinal Aβ and p-Tau IF scores

Age groups	Age (year)	Sex	Breed	Size	Ophthalmological disorders which resulted in enucleation	Pathological IF findings in the dog retina			
						$A\beta_{40}$ oligomers	$A\beta_{42}$ oligomers	Aβ	p-Tau
Young (1-5 years)	1	SF	Staffordshire terrier	Medium	Anterior segment dysgenesis	+	+	-	-
	1.4	M	Siberian husky	Medium	Anterior segment dysgenesis	-	-	-	-
	1.8	SF	Mixed breed	ND*	Anterior segment dysgenesis	+++	+++	-	-
	1.10	SF	Siberian husky	Medium	Anterior segment dysgenesis	+++	+++	-	-
	2	F	Chihuahua mix	Toy	Anterior segment dysgenesis	+++	+++	-	-
	2	M	Bichon frise mix	Small	Proptosis	+++	+++	-	-
	2	NM	Shih Tzu	Small	Melanoma limbal	++	++	-	-
	3	NM	German shepherd	Large	Rhabdomyosarcoma orbital	+++	+++	-	-
	3	F	Giant schnauzer	Large	Phthisis bulbi	++	++	-	-
	3.6	M	Siberian husky	Medium	Anterior segment dysgenesis	++	++	-	-
Middle age (6-10 years)	7	NM	Hound mixed	Medium	Hemangiosarcoma	-	-	-	-
	8	SF	Bedlington terrier	Small	Neoplasia	-	-	-	-
	8	SF	Cocker spaniel	Medium	Conjunctivitis	+++	+++	+	-
	8	M	Mixed breed	ND*	Conjunctival melanoma	+++	+++	+	-
	8	M	Cocker spaniel	Medium	Pre glaucoma	+++	+++	+++	-
	8	F	German shepherd mix	Large	Mast cell tumor	++	++	+	-
	8	SF	Great dane dog	Giant	Complex apocrine adenocarcinoma	+	+	-	-
	9	M	Jack Russell terrier dog	Small	Sialoadenitis	++	++	-	-
	9.5	SF	Boxer dog	Large	Hemangioma	+	+	-	-
	9.9	SF	Cocker spaniel	Medium	Adenocarcinoma a orbital	+++	+++	+++	-
Elderly (11-16 years)	11	NM	Mixed breed	ND*	Squamous cell carcinoma	-	-	-	-
	11	NM	Beagle	Small	Melanoma conjunctival	+++	+++	+++	-
	11.9	SF	Bouvier des flanders	Large	Melanoma eyelid	+	+	-	-
	12	SF	German shepherd	Large	Melanoma eyelid	+++	+++	+++	+++
	12	SF	German shepherd mix	Large	Melanoma conjunctival	+++	+++	+++	-
	12	NM	Husky mix	Medium	Orbital carcinoma	+++	+++	++	+++
	12.8	NM	Shih-tzu	Small	Glaucoma	+++	+++	++	+++
	13	NM	Border collie	Medium	Melanoma skin	+++	+++	+++	+++
	15	SF	Basset hound	Medium	Conjunctival hemangiosarcoma	+++	+++	+++	-
	16	SF	Shih Tzu	Small	Squamous cell carcinoma	+	+	-	-

Existing ophthalmological conditions of the 30 neurologically intact unimpaired dogs were determined from the clinical report. Sizes were determined according to the American Kennel Club (AKC). Size range between 34 - ≥ 54 kg is considered "giant", 24-38 kg is considered "large", 15-29 kg is considered "medium", 3-15 kg is considered "small", and 0.9-4 kg is considered "toy". Aβ oligomers Aβ plaques and p-Tau staining intensity were semi-quantitatively analyzed and scored across the retinal layers under the confocal microscope (LSM800, Zeiss, Oberkochen, Germany). The total area was examined at 40 magnification and categorized into no immunostaining "-"; low immunoreactivity found only in limited areas of the retinal layers "+", moderate immunoreactivity where deposits were more apparent "++"; and finally strong immunoreactivity with widespread Aβo, Aβp, and p-Tau labeling was exhibited "+++". ND*: Not determined; SF: spayed female; F: Female; NM: neutered male; M: male; IF: immunofluorescence.

incubated for 1 h at RT with the following secondary antibodies in PBS: HRP-conjugated anti-mouse IgG (Sigma-Aldrich, Missouri, United States) or anti-rabbit IgG (Sigma-Aldrich, Missouri, United States). The

sections were then washed three times with PBS before the addition of the 3,3'-Diaminobenzidine substrate chromogen system and incubated for 5-10 min. The sections were then counterstained with hematoxylin for 1 min before mounting. Sections were finally imaged using Olympus CX 43 light microscopy (Shinjuku, Tokyo, Japan).

The staining intensity was semi-quantitatively analyzed and scored across the retinal layers. Each bright field was examined at 40× magnification and categorized into no immunostaining "-"; low immunoreactivity, found in limited areas of the retinal layers "+"; moderate immunoreactivity where Aβ deposits were numerous "++"; and finally strong immunoreactivity with widespread A11 and 4G8 positive Aβ labeling "+++" [Table 1].

Immunofluorescence detection and co-localization studies

To investigate whether Aβo co-localized with Aβp or with p-Tau, we performed immunofluorescence double labeling using camelid-derived single domain anti-A_{40} (PRIOAD12) or anti-A_{42} (PRIOAD13) oligomer antibodies with 4G8 anti-Aβp antibody (Bio legend, San Diego, California, United States) or anti-phosphorylated tau AT8 antibody targeting amino acid residues Ser202/Thr205 (Thermo-fisher Scientific, Massachusetts, United States). Slides were processed as described above for IHC before adding the primary antibodies. The sections were double-stained with either PRIOAD 12 (1:500) or PRIOAD 13 (1:500)[57] and 4G8 antibody overnight (1:500) or AT8 (1:500) and either PRIOAD 12 (1:500) or PRIOAD 13 (1:500). Sections were then washed in Tris-buffered saline with 0.05% Tween 20 (TBST) and incubated with secondary antibodies at a dilution of 1:500, including goat anti-llama IgG conjugated to FITC (Bethyl Laboratories, Inc, Texas, USA) or donkey-anti-mouse IgG conjugated to Texas red (Sigma-Aldrich, Missouri, United States) for 2 hours at room temperature. Other sections were used as negative control and stained with secondary antibodies with the omission of the primary antibodies. Retinal sections derived from an APP/PS1 and TAU58/2 transgenic mice models[58,59] were used as positive control and stained with anti-Aβ and anti-P-Tau antibodies, respectively. The sections were covered, slipped with paramount aqueous mounting medium (Dako, Agilent, Santa Clara, California, United States), sealed, and dried overnight. Finally, the sections were visualized with LSM800 confocal microscope with a standard FITC/Texas Red double band-pass filter set (Zeiss, Oberkochen, Germany).

RESULTS

Histological assessment of the retina in 30 dogs

We performed H&E staining of the canine eye sections and examined the retinal layers to assess their general morphological appearance and identify pathological changes such as vacuolation, neuronal death, and eosinophilic deposits[49,55]. No specific lesions were observed in the retinal layers of the young and middle age groups (data not shown). However, scattered eosinophilic deposits in the ganglion cell layer (GCL) and inner nuclear layer (INL) of the retina were noticeable in 5/10 dogs in the older age group (11-16 years old), including an 11-year-old Beagle, a 12-year-old German shepherd, a 12-year-old Husky mix, a 12.8-year-old Shih Tzu, and a 15-year-old Basset hound [Figure 1A and B]. Furthermore, CR staining used to detect any amyloid fibrils and CAA in eye sections of the dogs did not display any staining in the retina tissues from all age groups (data not shown).

Immunohistochemical detection of Aβ oligomers and plaques in the retina of 30 dogs

While demonstrated in the CNS of aged animals including cats and bears[24,60], dogs have been more comprehensively studied and shown to develop human type Aβ deposition at a very young age. Retinal tissues stained with A11 exhibited intracellular Aβo depositions in the outer nuclear layer (ONL), INL, and GCL [Figure 1C and D]. The intraneuronal Aβo intensity following A11 staining ranged from low (+), moderate (++) to strong (+++) in all dogs in the young age group except a 1.4 years old male Siberian husky

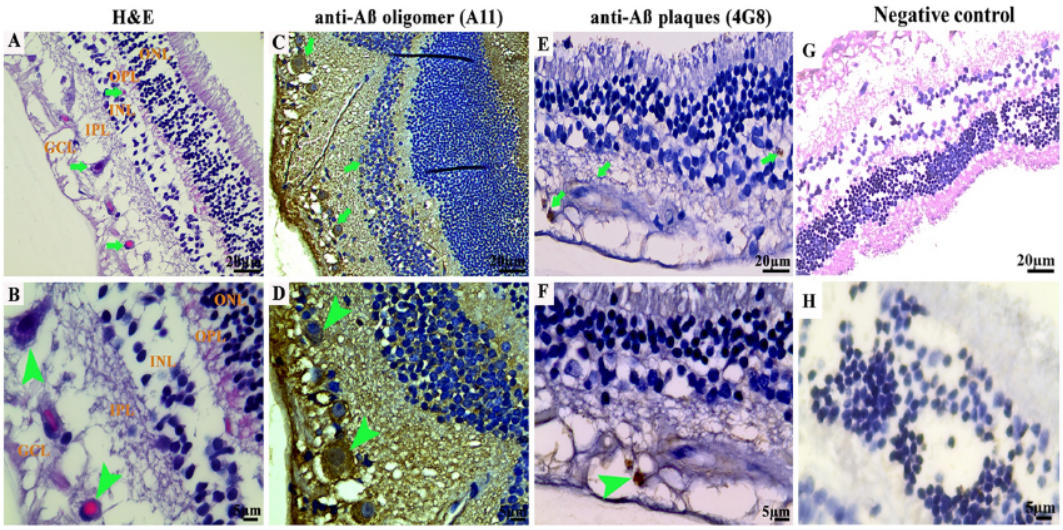

Figure 1. Photomicrographs of the microscopic lesions in the canine retina. The retina in a 12-year-old German shepherd dog. Eosinophilic deposits (green arrows) were observed in the ganglion cell layer (GCL) and inner nuclear layer (INL). Representative of 10 cases of old-aged dogs. Hematoxylin and eosin (H&E) 40×. (B) Higher magnification of the deposits in image (A) (green arrowhead) in the GCL. H&E 100×. (C) Retina of a 3-year-old German shepherd dog. Immunohistochemical staining with A11 anti-Aβo IgG antibody exhibited cytoplasmic Aβo depositions in the GCL and INL (green arrows). Representative of 10 cases of young age group. IHC 40×. (D) Higher magnification of deposits from image (C) in the GCL (green arrowheads). IHC 100×. (E) Retina of a 12-year-old German shepherd mix dog. Immunohistochemical staining with 4G8 anti-Aβp IgG antibody exhibited extracellular Aβ aggregates in the GCL and INL (green arrows). IHC 40×. (F) Higher magnification of deposit from image (E) in the GCL (green arrowhead). (G and H) Representative retinal sections derived from dogs of all age groups stained with secondary antibodies with the omission of the primary antibody did not show any depositions (40× and 100×, respectively). The photomicrographs were taken from the peripheral region of the retina - away from the optic disc. Representative of all 30 dogs examined. IHC 100×. IHC: Immunohistochemistry.

that did not display any A11 positive stain [Supplementary Figure 1A]. Of note, retinal sections derived from wild-type mice were used as negative control following immunofluorescence staining [Supplementary Figure 1B and C].

A spayed female mixed breed aged 1.8 years, a female Chihuahua aged 2 years, and a neutered male German shepherd aged 3 years showed strong (+++) accumulation of A11 positive Aβo [Figure 1C and D]. Two dogs displayed a low amount (+) of A11 positive Aβo in the retinal layers in the middle and old age groups, including an 8-year-old male Cocker spaniel and an 11.9-year-old spayed female Bouvier des Flanders [Table 1]. In addition, 4G8-positive small rounded and dot-like extracellular Aβp deposits were observed in the INL, inner plexiform layer (IPL), and GCL [Figure 1E and F] of the retina. They were structurally different from the typical large diffuse plaques normally observed in dog brains with CCD[21,30,61]. The extracellular Aβp intensity following 4G8 staining ranged from low (+) to moderate (++) in some dogs in the old age group, including a 15-year-old spayed female Basset hound, a 12.8-year-old neutered male Shih Tzu, and a 12-year-old spayed female German shepherd. Other dogs in this age group were all negative for 4G8 staining [Table 1, Figure 1E and F]. Representative retinal sections derived from dogs of all age groups stained with secondary antibodies with the omission of the primary antibody did not show any depositions [Figure 1G and H].

Immunofluorescence detection and co-localization of retinal $A\beta_{40}$, $A\beta_{42}$ oligomers, and Aβ plaques in the retina of 30 dogs

To confirm the presence of retinal $A\beta_{40}$ and $A\beta_{42}$ oligomers and to determine whether $A\beta_{40}$ and/or $A\beta_{42}$ oligomers co-localized with Aβp in the retinas of different age groups and breeds of neurologically intact dogs, we performed immunofluorescence double staining using PRIOAD12 ($A\beta_{40}$ oligomers), PRIOAD13

($A\beta_{42}$ oligomers) camelid-derived single domain anti-Aβ oligomer and 4G8 anti-Aβp antibodies. Morphologically, Aβo appeared as globular and annular in shape[55,62] and deposited intracellularly in the ONL, INL, and GCL [Figures 2-4]. 4G8 positive Aβ plaques appeared morphologically as dot-like and small rounded extracellular deposits[50,54] in the outer plexiform layer (OPL), IPL, and GCL [Figures 2-4]. We found that both $A\beta_{40}$ and $A\beta_{42}$ oligomers staining was widespread in the majority of the dogs in young [Table 2, Figure 2A and B], middle [Table 2, Figure 3A and B], and old age groups [Table 2, Figure 4A and B] except four dogs including a 1.4-years-old male Siberian husky, a 7-years-old neutered male Hound mixed, an 8-years-old spayed female Bedlington terrier and an 11-years-old mixed breed. In comparison, Aβp was absent in young dogs [Table 2, Figure 2C and D], but its presence in the middle age group was moderate [Table 2, Figure 3D and E] and conspicuous in the old age group [Table 2, Figure 4D and E]. Therefore, no co-localization of Aβo and Aβp was noticed in the retinal layers of the young age group [Figure 2E-H]. However, retinal Aβp was shown to co-localize with $A\beta_{40}$ or $A\beta_{42}$ in the GCL, IPL & INL of the middle [Figure 3G, H, J and K] and old [Figure 4G, H, J and K] neurologically intact age groups. Co-localization of Aβ oligomers and 4G8 positive Aβ plaques were also exhibited in the retinal vessel wall, where young dogs didn't exhibit any co-localization, the middle age group showed scarce co-localization [Figure 3I and L], and the older age group exhibited conspicuous co-localization in the vessel wall [Figure 4I and L]. In the middle age group, two Cocker spaniels aged 8 years male and 9.9 years female respectively exhibited widespread co-localization of Aβo and Aβp, and in the old age group, a neutered male Beagle aged 11 years, two spayed female German shepherds aged 12 years, a neutered male Border collie aged 13 years, and a spayed female Basset hound aged 15 years displayed strong and widespread co-localization of Aβo and Aβp. This co-localization study revealed an age-dependent distribution and co-accumulation of Aβo and Aβp in the retina of the neurologically intact dogs. We found that the young dogs exhibited widespread accumulation of $A\beta_{40}$ and $A\beta_{42}$ oligomers without any plaque deposits; in the middle age group, $A\beta_{40}$ and $A\beta_{42}$ oligomers accumulation was less conspicuous and, in some cases, co-localized with Aβp; and at the old age group, there was widespread and strong co-localization of Aβo and Aβp strongly distributed in most dogs [Supplementary Figures 2 and 3]. Representative retinal sections derived from dogs of all age groups stained with secondary antibodies with the omission of the primary antibody did not show any co-localization [Supplementary Figure 4A-F]. Retinal sections derived from an APP/PS1 mouse were used as positive control and confirmed the presence of $A\beta_{40}$ and $A\beta_{42}$ oligomers [Supplementary Figure 5A and B] and Aβp [Supplementary Figure 5C][49,55].

Immunofluorescence co-localization of retinal Aβ oligomers and phosphorylated tau in the retina of 30 dogs

To confirm the presence of p-Tau and to investigate whether p-Tau co-localizes with Aβo, we performed double fluorescence staining of Aβo and p-Tau using PRIOAD 12 ($A\beta_{40}$) or PRIOAD 13 ($A\beta_{42}$) anti-oligomer[57] and AT8 anti-p-Tau antibody, targeting Ser202/Thr205[54]. Aβo appeared as globular and annular in shape[55,62] [Figure 5A and B] and AT8 positive p-Tau appeared morphologically as diffuse and granular intracellular deposits[54] in the OPL, INL, IPL, and GCL [Figure 5C and D]. Overall, Aβo and p-Tau did not display consistent co-localization in all age groups, as P-tau was only detected in the old age group and $A\beta_{40}$ or $A\beta_{42}$ oligomers were identified in all age groups. Among ten dogs in the old age group, only four dogs have displayed the presence of p-Tau including a spayed female German shepherd and a neutered male Husky aged 12 years, a neutered male Shih Tzu aged 12.8 years, and a neutered male Border collie aged 13 years, and they exhibited strong co-localization with $A\beta_{40}$ or $A\beta_{42}$ oligomers [Figure 5E-H]. Representative retinal sections derived from Tau 58/2 mouse were used as positive control and confirmed the presence of AT8 positive p-Tau [Supplementary Figure 5D][58]. Representative retinal sections derived from dogs of all age groups stained with secondary antibodies with the omission of the primary antibody did not show any co-localization [Supplementary Figure 6A and B].

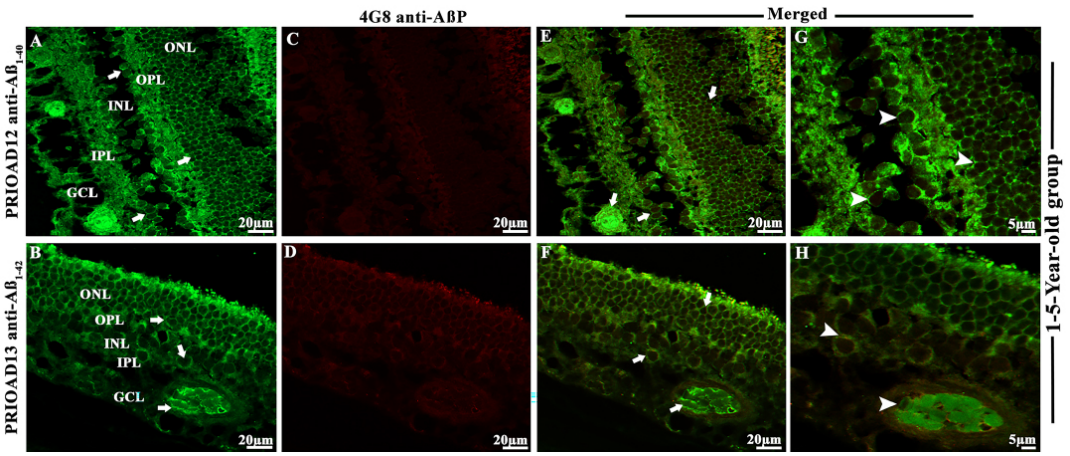

Figure 2. Immunofluorescence (IF) detection and co-localization of retinal amyloid-beta oligomers and amyloid-beta plaques in the dogs of the 1-5-year-old group. Retinal co-staining of oligomers with anti-$A\beta_{40}$ (PRIOAD12) or anti-$A\beta_{42}$ (PRIOAD 13) camelid-derived single domain antibodies (green) and plaques with anti-Aβ (4G8) antibody (red) of a 3-year-old German shepherd dog (A-H). A and B show widespread accumulation of $A\beta_{40}$ and $A\beta_{42}$oligomers in the GCL, INL, and ONL (white arrows) and retinal vasculature, respectively. IF 40×. (C and D) No Aβp was detected in the retinal layers. IF 40×. (E and F) Co-localization of Aβo and Aβp was not observed in the retinal layers of this animal (Aβo was present - white arrows). G and H are higher magnification 100× of images (A and B) in the GCL and INL, ONL and retinal vasculature. Representative of 10 dogs in the younger age group (1-5 years). GCL: Ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

Influence of demographic factors on the retinal deposition of Aβ oligomers, Aβ plaques, and phosphorylated tau in 30 dogs

To investigate the influence of the demographic factors on retinal Aβ and p-Tau deposition, staining intensity was compared with the age, breed, size, and sex of the neurologically intact dogs [Table 2]. $A\beta_{40}$ and $A\beta_{42}$ oligomers, Aβp and p-Tau fluorescence intensity, were assessed at 40× magnification. Immunoreactivity throughout the retinal layers was semi-quantified and categorized into no immunostaining "-"; low immunoreactivity, exhibited in limited areas of the retinal layers "+"; moderate immunoreactivity where deposits were more apparent "++" and finally strong immunoreactivity with widespread Aβo, Aβp and p-Tau labeling "+++" [Table 2]. After comparing the age of the dogs and immunofluorescence staining scores, we found that both $A\beta_{40}$ and $A\beta_{42}$ oligomers staining was high in young dogs, which slightly decreased in the middle age group, then finally, an upward trend was noticed in older dogs [Table 2, Figure 6]. In comparison, Aβp was absent in young dogs, but its presence in the middle age group was moderate and high in the old age group [Table 2, Figure 6]. Finally, p-Tau deposits were not observed in the young and middle age groups, whereas old dogs exhibited widespread staining for p-Tau [Table 2, Figure 6]. However, when the size of the dog was compared with the pathological outcome, we found that six medium-sized dogs displayed strong Aβo staining and only two dogs exhibited strong p-Tau staining [Table 2, Figure 7]. In addition, seven dogs revealed strong Aβp staining, of which four were medium size [Table 2, Figure 7].

Influence of eye pathology on the retinal deposition of Aβ oligomers, Aβ plaques, and phosphorylated tau in 30 dogs

Further, to understand whether the presence of pre-existing underlying eye pathology in the dogs may influence retinal Aβ and p-Tau depositions, we compared their staining intensity with the reported underlying clinical eye conditions [Table 2]. We found that four young dogs aged 1-5 years affected with anterior segment dysgenesis, a spectrum of disorders that affect the development of the anterior segment, including the cornea, iris, ciliary body, and lens[63-65], displayed "moderate" to "strong" intensity staining for $A\beta_{40}$ and $A\beta_{42}$ oligomers [Table 2]. However, another two dogs in this age group and affected with anterior segment dysgenesis showed little to no deposition of $A\beta_{40}$ and $A\beta_{42}$ oligomers [Table 2]. Eighteen out of

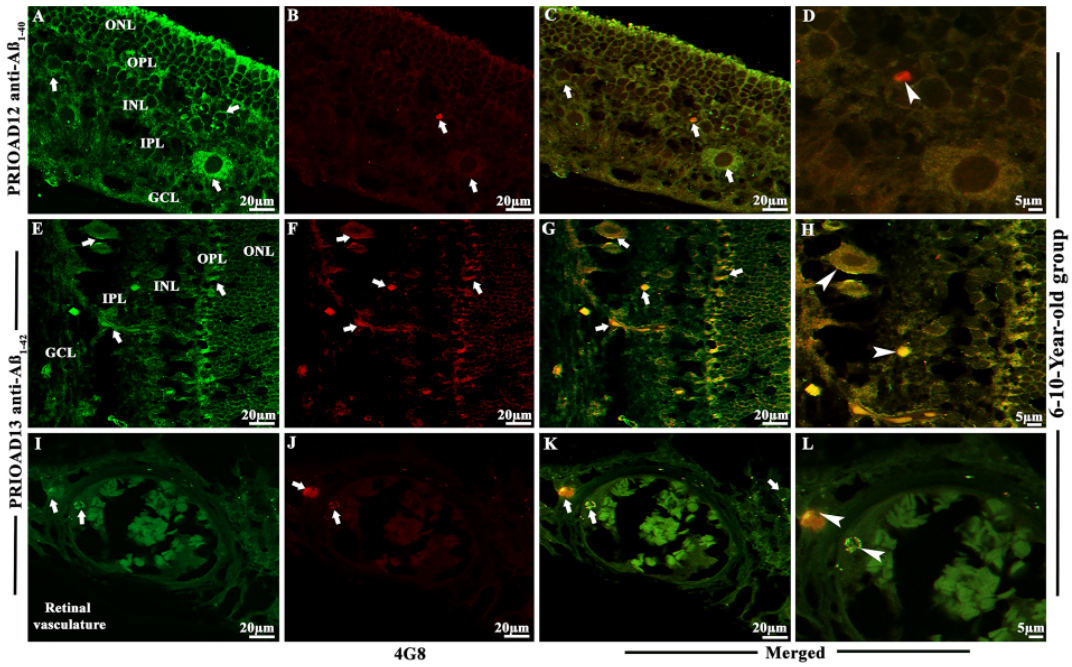

Figure 3. Immunofluorescence detection and co-localization of retinal amyloid-beta oligomers and amyloid-beta plaques in dogs of the 6-10-year-old group. Retinal co-staining with anti-$A\beta_{40}$ (PRIOAD 12) and anti-$A\beta_{42}$ (PRIOAD 13) camelid-derived single domain antibody (green) and 4G8 antibody (red) of a 9-year-old Cocker spaniel dog (A-L). (A) A large number of $A\beta_{40}$ and (B) $A\beta_{42}$ oligomers were found in the GCL, INL, and ONL (white arrows, 40×). (C) Detection of $A\beta_{42}$ oligomers in the vasculature. 4G8 positive Aβ plaque-like deposits were observed in the (D and E) GCL, IPL, INL, and OPL of the retina (white arrows, 40×). Widespread co-localization was observed in the (G and H) retinal layers (white arrows, 40×). Co-localization of 4G8 positive Aβ plaque with (J) $A\beta_{40}$ and (K) $A\beta_{42}$ depositions (white arrowhead) showed with higher magnification (100×) in the GCL and INL of the same 9-year-old Cocker spaniel dog retinal section. (C) Aβ oligomers and (F) 4G8 positive Aβ plaques were observed in the retinal vasculature (white arrows). (I and L) Co-localization of Aβ oligomers and 4G8 positive Aβ plaques were exhibited with 40× and with higher 100× magnification in the retinal vessel wall, respectively (white arrowhead). The photomicrograph was derived from the peripheral region of the retina - away from the optic disc. Representative of 10 dogs examined from middle age group (6-10 years). GCL: Ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

thirty dogs presented with eye neoplasms, including two in the young age group, seven in the middle age group, and nine in the old age group [Table 2]. There was no clear relation between neoplasms and the intensity of Aβ or p-Tau [Table 2]. Interestingly, eye inflammation (proptosis/ phthisis bulbi in young dogs and conjunctivitis in a middle-aged dog) corresponded to $A\beta_{40}$ and $A\beta_{42}$ oligomers intensity that ranged from "moderate" to "strong" [Table 2]. Finally, a case of pre-glaucoma in the middle age group and a case of glaucoma in the old age group also matched well with $A\beta_{40}$ and $A\beta_{42}$ oligomers as well as Aβp intensity. Overall, this analysis appears to indicate that no direct influence of pre-existing eye disorders on Aβ and p-Tau intensity exists; however, the small size of the cohorts used in this study did not allow to reach a substantive conclusion, and studies with much larger cohorts are needed.

DISCUSSION

Eye imaging can provide an opportunity to develop an easily accessible and point-of-care routine diagnostic testing to help predict MCI/AD early[66,67]. A study by Ko and colleagues examined the retinal NFL thickness and cognitive status of individuals aged 40 to 69 years over three years[68]. The authors found that individuals with thinner NFL showed a higher incidence of reduced cognition.

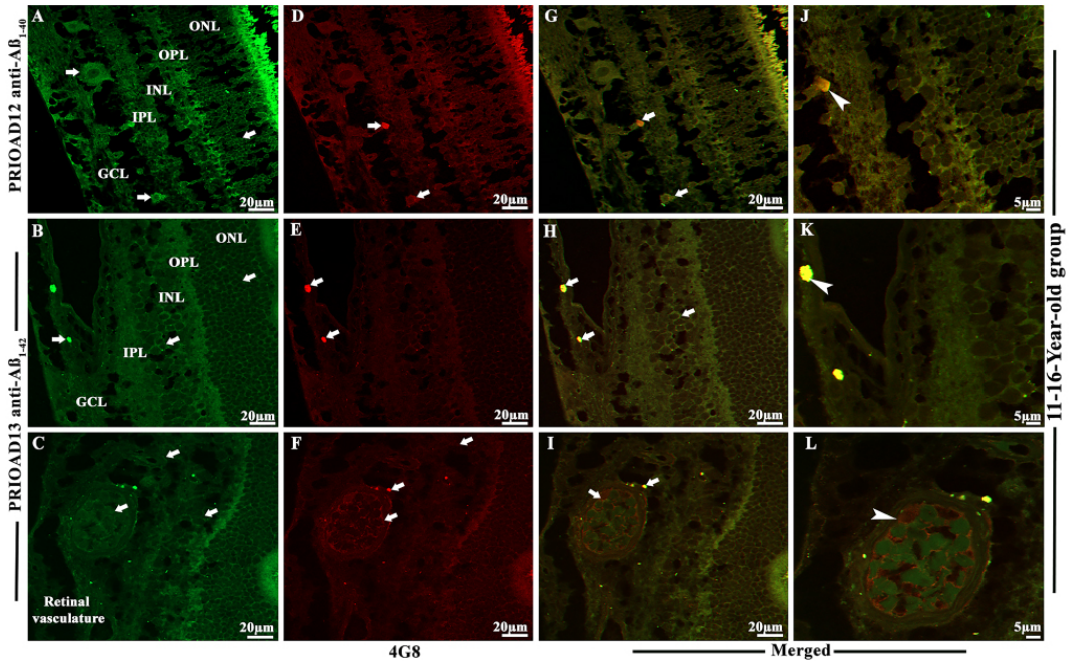

Figure 4. Immunofluorescence co-localization of retinal amyloid-beta oligomers and amyloid-beta plaques in dogs of 11-16-year-old group. Retinal co-staining with anti-$A\beta_{40}$ (PRIOAD 12) and anti-$A\beta_{42}$ (PRIOAD 13) camelid-derived single domain antibody (green) and 4G8 antibody (red) of a 12-year-old German shepherd dog (A-L). A large number of (A) $A\beta_{40}$ and (B) $A\beta_{42}$ oligomers were found in the GCL, INL, and ONL (white arrows, 40×). 4G8 positive Aβ plaque-like deposits were observed in the (D and E) GCL of the retina (white arrows, 40×). Widespread co-localization was observed in the (G and H) retinal layers (white arrows, 40×). Co-localization of 4G8 positive Aβ plaque with (J) $A\beta_{40}$ and (K) $A\beta_{42}$ depositions (white arrowhead) showed with higher magnification (100×) in the GCL of the same 12-year-old German shepherd dog retinal section. (C) Aβ oligomers and (F) 4G8 positive Aβ plaques were observed in the retinal vasculature (white arrows). (I and L) Co-localization of Aβ oligomers and 4G8 positive Aβ plaques were exhibited with 40× and also with higher 100× magnification in the retinal vessel wall, respectively (white arrowhead). The photomicrograph was derived from the peripheral region of the retina - away from the optic disc. Representative of 10 dogs examined from elder age group (11-16 years). GCL: ganglion cell layer; INL: inner nuclear layer; ONL: outer nuclear layer.

Despite the validation of the dog as a robust translational model for AD[24,30,69], only very limited studies investigated AD-related changes in the young and neurologically intact dogs. One study by Stylianaki and colleagues confirmed a similar pattern to that of humans; 61 dogs were subdivided into young (0-4 years old), middle-aged (4-8 years old), aged cognitively normal (8-20 years old), and aged cognitively impaired (8-17 years-old)[70]. The authors found that young dogs displayed the highest levels of total plasma $A\beta_{42}$ and $A\beta_{42}/A\beta_{40}$ ratio and the middle-aged dogs had the highest cerebrospinal fluid $A\beta_{40}$ and $A\beta_{42}$ when compared to neurologically intact aged dogs. Our first step was to investigate the distribution and morphological appearance of both $A\beta_{40}$ and $A\beta_{42}$ oligomers representing the canine lifetime, in the retinal layers of these neurologically intact 30 dogs. Previous studies have shown that A11 anti-oligomer antibody binds to different epitopes of the Aβo but also reacts with oligomeric aggregates of other proteins independent of their primary sequences. Of note, A11 cannot differentiate between $A\beta_{40}$ and $A\beta_{42}$. In contrast, PRIOAD12 and PRIOAD13 nanobodies bind to $A\beta_{40}$ and $A\beta_{42}$, respectively. Previous studies reported an inverse interrelationship between neurotoxicity and the size of Aβo, where the toxic effect of Aβo was shown to decrease with increased size. The general molecular weight of Aβo was found to range between 10-100 kDa in AD brain and included dimers to dodecamer[71]. A study by Lambert and colleagues first describes the cytotoxic effect of the small diffusible Aβo on the hippocampal neurons[72]. The small Aβo referred to as Aβ-derived diffusible ligands toxicity was tested in organotypic mouse brain slice cultures. The authors found that 17 and 22 kDa small oligomers killed hippocampal neurons at nanomolar concentration[72]. In addition, a study by Cizas and colleagues confirmed that oligomers larger than 30 KDa have a less toxic effect on the

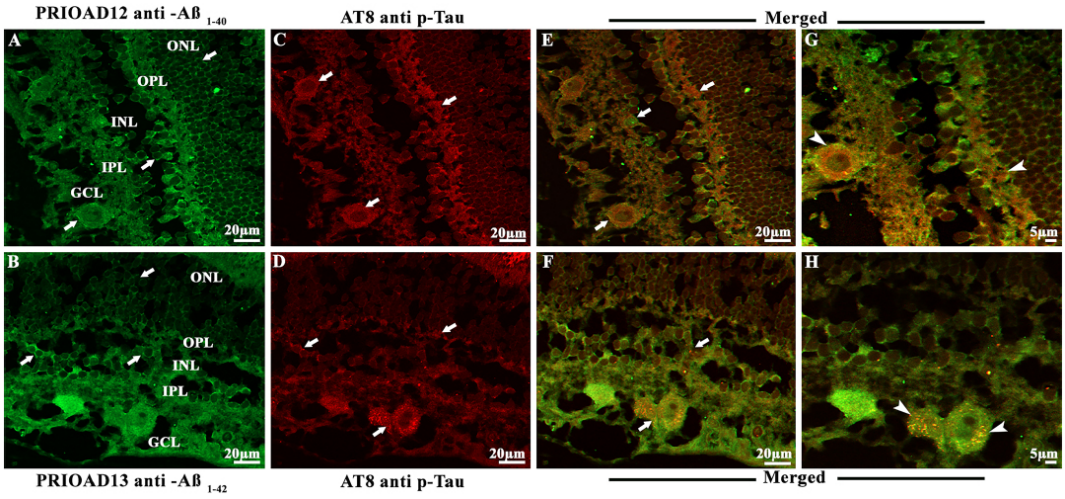

Figure 5. Immunofluorescence co-localization of retinal amyloid-beta oligomers and hyperphosphorylated tau in dogs of 11-16-year-old group. Retinal co-staining with anti-$A\beta_{40}$ (PRIOAD 12) and anti-$A\beta_{42}$ (PRIOAD 13) camelid-derived single domain antibody (green) and AT8 antibody (red) of a 12-year-old German shepherd dog. A large number of (A) $A\beta_{40}$ and (B) $A\beta_{42}$oligomers were found in the GCL, INL, and ONL (white arrows, 40×). AT8 positive diffuse p-Tau-like deposits were observed in the (C and D) GCL, IPL, INL, and OPL of the retina (white arrows, 40×). Widespread co-localization was observed in the (E and F) retinal layers (white arrows, 40×). Co-localization of AT8 positive p-Tau with (G) $A\beta_{40}$ and (H) $A\beta_{42}$ depositions (white arrowhead) showed with higher magnification (100×) in the GCL and OPL of the same 12-year-old German shepherd dog retinal section. The photomicrograph was derived from the peripheral region of the retina - away from the optic disc. Representative of 10 dogs examined from elder age group (≥ 11 years). GCL: Ganglion cell layer; IPL: inner plexiform layer; INL: inner nuclear layer; OPL: outer plexiform layer; ONL: outer nuclear layer.

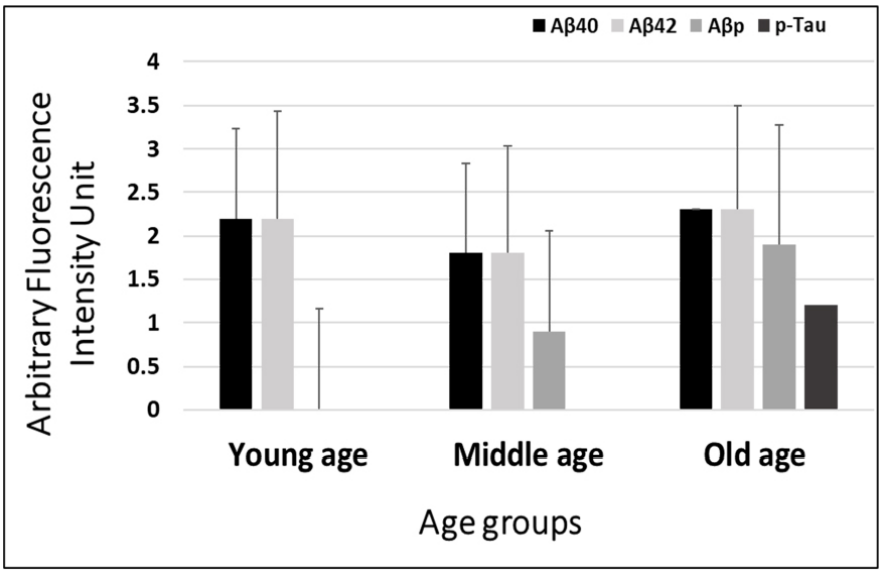

Figure 6. Semiquantitative analysis of $A\beta_{40}$ and $A\beta_{42}$ oligomers, Aβ plaques, and p-Tau in neurologically intact young (1-5-years), middle (6-10-years), and old (11-16-years) age groups of dogs. PRIOAD12 ($A\beta_{40}$ oligomers), PRIOAD13 ($A\beta_{42}$ oligomers), 4G8 (Aβp), and AT8 (p-Tau) fluorescence immunoreactivity and intensities were examined and quantified at 40× magnification under the fluorescence microscope. Semiquantitative analyses were compared with the three different age groups. A large amount of $A\beta_{40}$ and $A\beta_{42}$ oligomers were found in young dogs, which decreased in middle age group, then finally, an upward trend was noticed in older dogs. In comparison, Aβp were completely absent in young dogs, and then moderately found in middle, and large amounts in old age groups. Finally, p-Tau deposits were not found in young and middle age groups of dogs, whereas old dogs exhibited a considerable amount of p-Tau.

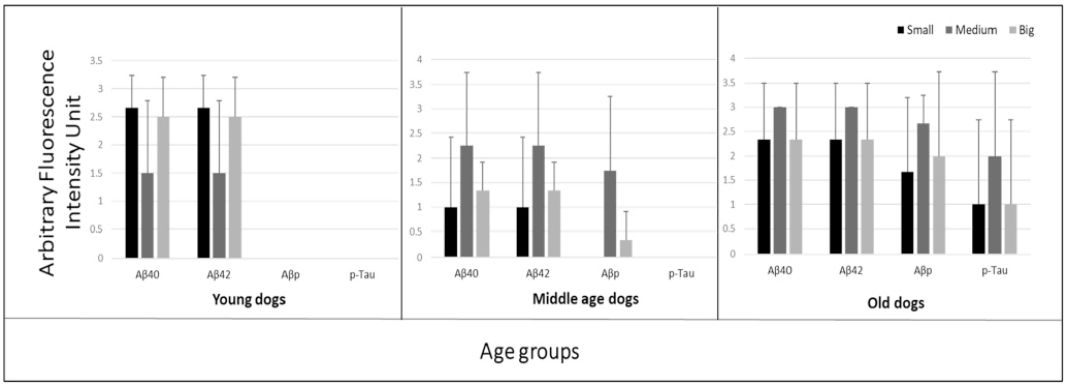

Figure 7. Influence of the size of dogs on the retinal deposition of amyloid-beta oligomers (Aβo), plaques (Aβp), and phosphorylated tau (p-Tau) in cognitively unimpaired young (1-5-years), middle (6-10-years) and old (11-16-years) age groups of dogs. PRIOAD12 ($Aβ_{40}$ oligomers), PRIOAD13 ($Aβ_{42}$ oligomers), 4G8 (Aβp), and AT8 (p-Tau) fluorescence immunoreactivity and intensities were examined and quantified at 40× magnification under the fluorescence microscope. In three different age groups, semiquantitative analyses were compared with the size of the dogs. In young dogs, a large number of oligomers were displayed by the small and big size dogs. The majority of medium-sized dogs displayed strong Aβo and Aβp staining intensity in the middle-aged dogs. Finally, among the different sizes of old dogs, medium-sized breeds displayed the highest amount of Aβo, Aβp, and p-Tau staining intensity.

inhibition of long-term potentiation[73]. They also suggested the transition of sizes from small to large correlate with the high to a low toxic effect of Aβo[73]. A11 specifically binds to the prefibrillar oligomers. However, studies suggested that the specificity of A11 to AD-related oligomers might vary as it recognized prefibrillar oligomers from various proteins that share a common structure including α-synuclein, islet amyloid polypeptide, polyglutamine (PolyQ), lysozyme, and prion peptide[36]. A study by Glabe and colleagues reported the ideal band size of prefibrillar oligomer-specific antibody A11 ranged from approximately tetrameric up to ~75 kDa[35]. In our study, we confirmed the presence of an A11-specific band at ~70KDa, confirming binding to prefibrillar oligomers in APP/PS1 mice. In comparison, camelid-derived single-domain nanobodies bind to ~15KDa band representing the small oligomers. In agreement with this current study, our previous report[55] also suggested that nanobodies were able to detect the toxic small diffusible oligomers specific to AD, whereas A11 binds to the larger oligomers believed to be less toxic to the neurons.

Studies suggested that in the canine brain, Aβo may be the toxic species responsible for cognitive decline and can potentially be an early biomarker for the detection of CCD[23,74]. Naaman and colleagues recently reported that $Aβ_{42}$ oligomers cause extensive retinal neurotoxicity in rats when compared to fibrillary $Aβ_{40}$ and $Aβ_{42}$[75]. Our findings demonstrated extensive deposition of $Aβ_{40}$ and $Aβ_{42}$ oligomers in the retinal layers, including GCL, INL, and ONL in 26/30 in the young, middle, and old age groups, except for a 1.4-year-old male Siberian husky, a 7-year-old neutered male Hound mixed, an 8-year-old spayed female Bedlington terrier, and an 11-year-old neutered male mixed breed. Moreover, we did not notice any difference in the intensity of immunofluorescence (graded - to +++), the pattern of deposition, or morphology between $Aβ_{40}$ and $Aβ_{42}$ oligomers in all age groups. This presentation was unlikely influenced by the breed of the animals; for instance, out of three Siberian Huskies, one dog failed to display $Aβ_{40}$ and $Aβ_{42}$ oligomers accumulation. Remarkably, the younger age group, with an age range of 1-5 years, equivalent to humans aged 15-40 years according to the American Kennel Club[76], has displayed extensive deposition of Aβ oligomers. Of interest, a 2013 brain study investigated the presence of three different types of Aβ oligomers, including Aβ trimers, Aβ*56, and Aβ dimers, in 75 cognitively unimpaired individuals aged 1 to 96 years[14]. Young children and adolescents were positive for Aβ oligomers; the authors found an age-related accumulation of Aβ oligomers where the level of the amyloid-β dimer was significantly higher in subjects in their 60s, and amyloid-β trimer

in their 70s, whereas Aβ*56 level was significantly higher in individuals in their 40s. The investigators proposed that Aβo, specifically Aβ*56, might trigger the pathological cascade in asymptomatic AD, a phase that may be identifiable two decades before the clinical onset[14].

Having found that the eyes of 26/30 dogs contained $A\beta_{40}$ and $A\beta_{42}$ oligomers, our next step was to demonstrate the presence/deposition of fibrillar Aβ in the canine retina of this same cohort. We show that none of the young dogs displays 4G8-positive Aβ fibrils and plaques (Aβp). However, Aβp were observed in some of the middle age group, where three dogs exhibited "low" staining intensity and two exhibited "strong" staining intensity in the GCL, IPL & INL. Aβp were also observed in the old age group, where five dogs showed "strong" signal intensity and two with "moderate" signal intensity in the GCL, IPL & INL. A pattern, not surprising, emerges; $A\beta_{40}$ and $A\beta_{42}$ oligomers are deposited in the retina following birth and subsequently, a fibrillar Aβ configuration, aggregated as plaques follow. Several studies have demonstrated the presence of Aβp in the canine brain and suggested only a weak correlation with the severity of cognitive dysfunction[77,78]. Schütt *et al.* investigated this question in further depth by studying dogs at differing ages, specifically aged 9-15 years, including neurologically intact dogs and dogs with CCD, and compared their amyloid burden with that of young dogs aged less than 6 years[69]. The authors reported that the levels of Aβ deposition strongly correlated with the age of the dog but not to their cognitive capacity. Asking the same questions regarding amyloid accretion in non-CNS neuronal populations, such as the retina, has not been investigated in the canine, but some recent reports confirmed their presence in the retina of AD[50,54,79]. In this study, we have demonstrated accumulation of retinal Aβp in the mature to an aged group of these 30 dogs which supports the hypothesis that Aβp might not influence the severity of cognitive deficits but can be a predictor of AD development[11,17]. Overall, our study revealed that the Aβ deposition pattern in the retina was such that Aβo was observed in all age groups, whereas Aβp accumulation was restricted to the middle and more intensely the old age group of dogs, regardless of demographic criteria, breed and gender. Interestingly, co-accumulation of both Aβo and Aβp were apparent in some middle and old dogs. This is in agreement with our report this year, where 17-18 months old APP/PS1 mice showed high levels of oligomers deposits in the retinal layers that co-localized with Aβp[55]. Previous neuropathological studies in AD have shown that cerebral Aβo is usually present in early disease stages and is the most cytotoxic of Aβ species, mostly responsible for neurotoxicity and synaptic dysfunction[34,80-83], whereas extracellular Aβp is believed to accumulate at later ages and may act as a reservoir for Aβo[84]. Moreover, the involvement of Aβo in retinal degeneration in AD has also been reported[85-87]. In the study reported herein, we noticed widespread distribution of Aβo in the retinal layers of dogs of all age groups, indicating potential involvement in retinal degeneration, perhaps leading to vision impairment in some dogs[88,89]. Of importance, a previous study by Ozawa and colleagues that focused on web and paper-based surveys of dogs aged ≥ 10 years to identify physical disturbances related to CCD showed that more than 90% of dogs affected with CCD had vision impairment[90]. In addition to the neural retina, we observed Aβ deposition s in the retinal microvasculature of young, middle, and old age groups, which might imitate CAA in AD[91-93]. Sharafi *et al.* suggested that retinal vasculature changes captured by hyperspectral imaging can differentiate cerebral amyloid status between cognitively impaired and unimpaired individuals[94].

Central to AD pathogenesis is the intraneuronal deposition of hyperphosphorylated tau (p-Tau) in granular form and eventual organization as NFTs, one of the cardinal neuropathological hallmarks[3,4]. In dogs with CCD, p-Tau, unlike NFTs, has been identified in the brain, but until recently, only in some cases. We speculate that, unlike human AD, p-Tau is only evident in pretangle granular form in dogs due to their shorter lifespan (Tayebi & Habiba, unpublished observation). p-Tau neuropathology was shown to develop about a decade before the formation of Aβp in AD brains and was hypothesized to trigger AD[95,96]. However, Aβo accumulation was shown to precede and drive p-Tau accumulation and transneuronal spread across synapses in the parietal cortex of AD[96].

In the retina of human AD patients, p-Tau displayed a diffuse pattern in the plexiform layers in the absence of NFTs[54]. Schön and colleagues reported the presence of AT8-positive intracellular NFTs and diffuse p-Tau signals in the retina of 5/6 deceased AD patients[97]. In this study, we investigated the presence of p-Tau Ser202/Thr205 to confirm the presence of retinal p-Tau in dogs. We revealed diffuse p-Tau distribution in the OPL, INL, IPL, and GCL in 4/10 dogs of the old age group, including a spayed female German shepherd and a neutered male Husky aged 12 years old, a neutered male Shih Tzu aged 12.8 years old, and a neutered male Border collie aged 13 years old. The same dogs also showed the widespread distribution of $A\beta_{40}$ and/or $A\beta_{42}$ oligomers and 4G8- positive Aβp. Similarly, in the brains of cognitively impaired, 14-17 years old dogs, Abey and colleagues have demonstrated the presence of p-Tau Ser202/Thr205 and p-Tau Ser396 in 1/6 and 6/6, respectively[40]. This pattern of deposition strongly supports a possible age-dependent progression as observed in AD mice models[49,55]. Previous studies have shown that breed variance does not determine the pathological outcome associated with CCD in aged dogs[98,99]. In agreement, our study did not reveal any breed-dependent pathological accumulation.

While previous studies have demonstrated the presence of Aβp and p-Tau in the aged dog brain[30,43], Pugleise *et al.* proposed that the acquisition of diffuse Aβp and p-Tau are unrelated and independent events of aged dogs[42]. Whether these two "ingredients" of AD dementia or CCD are closely linked (co-localized or at least act in concert) is likely crucial. In AD, it was shown that p-Tau and Aβo cause neuronal toxicity and may act synergistically to trigger synaptic dysfunction[36,37,100]. Manczak and colleagues showed that $A\beta_{40}$ or $A\beta_{42}$ oligomers co-localized with p-Tau in the brains of AD patients[101]. The authors also reported that the interaction between Aβo and p-Tau became more prominent with disease progression and was more pronounced at Braak stage V and VI compared to Braak stage III and IV[102,103]. The evidence from this canine retina study would support an interaction: we also show that p-Tau co-localized with $A\beta_{40}$ or $A\beta_{42}$ oligomers in the OPL, INL, IPL, and GCL of the retina of 4/10 dogs in the eldest age group. A recent PET brain imaging study by Lockhart *et al.*, which demonstrated the presence of both Aβ and Tau pathology in cognitively normal older adults, showed a significant spatial correlation with Aβ and Tau deposition[104]. Thus co-localization, while evident, does not itself necessarily imply neuronal cytotoxicity.

Several studies in the field of AD research highlighted the importance of diagnostic and therapeutic interventions in the asymptomatic phase while the individuals are cognitively intact[14,105]. However, it is challenging to first identify and then track disease progression in humans without sensitive, specific, and cost-effective early diagnostic approaches and a lack of robust natural translational models. In that context, dogs have the potential to be an effective natural model for the study of aging and AD. They share the same environment as humans[106,107]; there exists the option of brain CT and MRI imaging and intracranial biopsy, ease of cognitive testing which helps reduce physiological stress, and finally, a short lifespan[108]. Canine ophthalmology is a well-established clinical and investigative discipline (inherited retinopathies, for example). The current study provides strong impetus to track how this seemingly common process evolves, with potential consequences for aging, neurodegenerative disease, and also vision.

Lateralization in retinal and cerebral Aβ levels was previously investigated in an AD mouse model[109]. Some studies revealed left posterior brain-dominant lateralization in $A\beta_{42/40}$; however, no left or right brain hemisphere or retinal dominance lateralization was observed for $A\beta_{40}$ and $A\beta_{42}$. Although our current study does not provide information about left versus right retinal accumulation of Aβ, the significance of the lateralization in human Alzheimer's and CCD remains unknown. Moreover, previous reports have shown that Aβ accumulation was more prominent in the far-peripheral and mid-peripheral of the superior

temporal quadrant than in the central retina in AD patients[50,79]; however, in our study, no notable differences in Aβ and tau deposition have been observed in different parts of the retina.

The canine eye tissues used in this study had been surgically removed in the course of managing spontaneous disorders of the globe, eyelids, or structures within the orbit which necessitated enucleation. It could be asked whether any of these conditions played any part in Aβ and/or p-Tau deposition in the retina. We think this unlikely, given the diverse clinical conditions involved, which ranged from developmental abnormalities, inflammatory conditions, and proptosis to various neoplasms of differing grades of the eye, while other cases were disorders arising in the conjunctiva or orbit. The span of ages in these dogs (from juvenile to quite aged) would further make this seem unlikely and some evidence of human retinal Aβ and/or p-Tau exists, though such explorations are largely performed only at the end of life.

DECLARATIONS

Authors' contributions

Performed experiments and revised manuscript: Habiba U
Revised manuscript: Morley J, Krockenberger M
Was a major contributor in writing and revising the manuscript: Summers BA Conceived and designed experiments and wrote manuscript: Tayebi M
All authors read and approved the final manuscript.

Availability of data and materials

Not applicable.

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Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Sections of eyes used in this study were prepared from archived cases in the Comparative Ocular Pathology Laboratory of Wisconsin (COPLOW) at the Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin, Madison. Such tissues, historically submitted for disease investigation purposes, are not subject to approval by institutional animal ethical regulations..

Consent for publication

Not applicable.

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