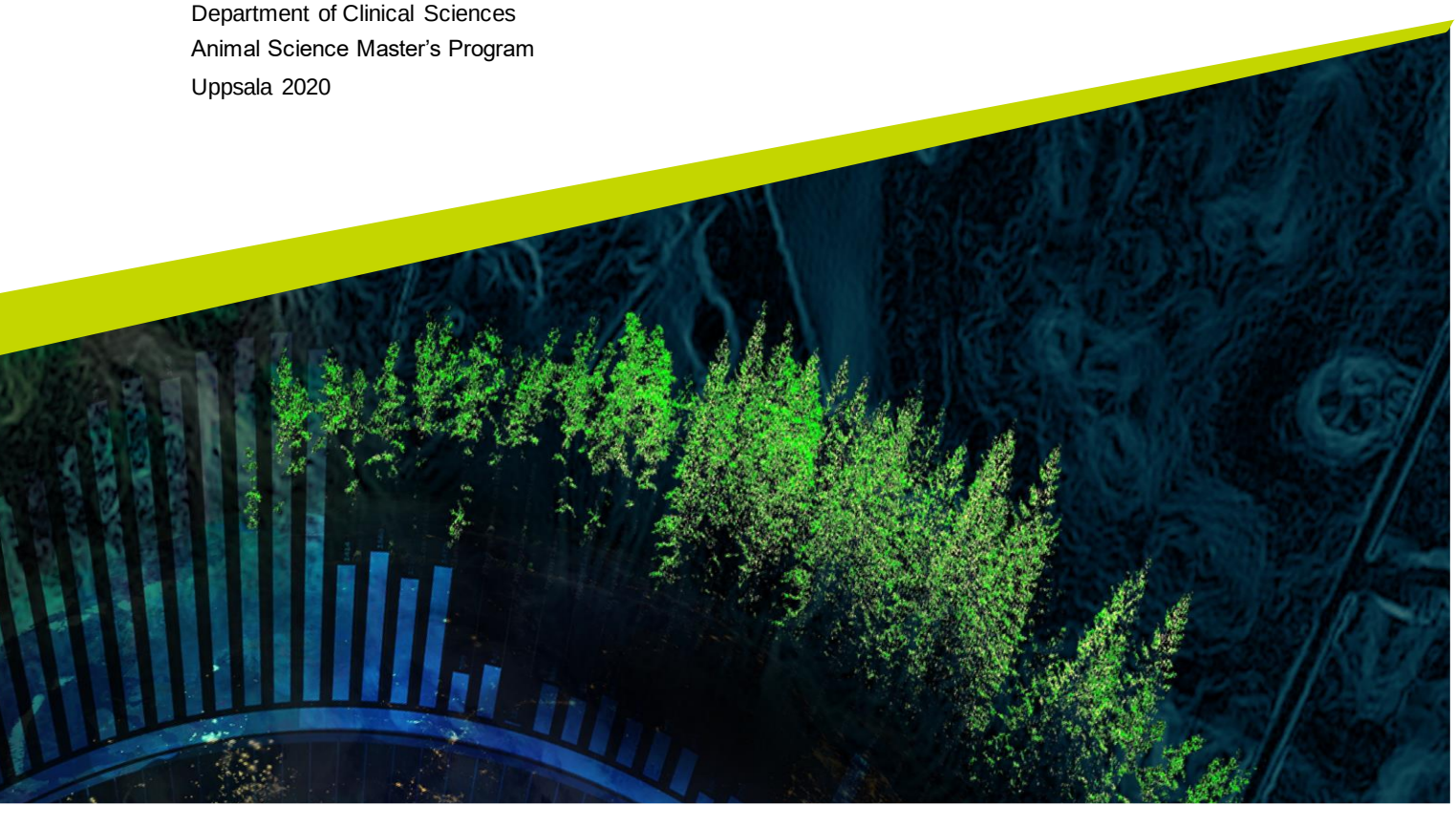
RELATION BETWEEN RETINAL MORPHOLOGY, VISUAL PATHWAY INTEGRITY AND PLASMATIC AMYLOID BETA 42 PEPTIDE IN OLD AND YOUNG BEAGLES

Emilia Miranda Peluso

Independent project 30 hp
Swedish University of Agricultural Sciences, SLU
Department of Clinical Sciences
Animal Science Master's Program
Uppsala 2020



Relation between retinal morphology, visual pathway integrity and plasmatic amyloid beta 42 peptide in old and young beagles

Emilia Miranda Peluso

Supervisor: Björn Ekesten, SLU, Department of Clinical Sciences
Assistant supervisor: Anna Svensson, SLU, Department of Clinical Sciences
Examiner: Lena Ström, SLU, Department of Clinical Sciences

Credits: 30 hp
Level: Second cycle, A2E
Course title: Independent Project in Animal Science
Course code: EX0870
Programme/education: Animal Science Master's program

Place of publication: Uppsala
Year of publication: 2020
Online publication: http://stud.epsilon.slu.se

Keywords: Alzheimer, amyloid beta, cognitive dysfunction, dogs, optical coherence tomography, visual evoked potentials.

Sveriges Lantbruksuniversitet
Swedish University of Agricultural Sciences

Faculty of Veterinary Medicine and Animal Science
Department of Clinical Sciences

Publishing and archiving

☒ YES, I/we hereby give permission to publish the present thesis in accordance with the SLU agreement regarding the transfer of the right to publish a work.

☐ NO, I/we do not give permission to publish the present work. The work will still be archived and its metadata and abstract will be visible and searchable.

Abstract

The spontaneously occurring behavioural syndrome referred to as canine cognitive dysfunction (CCD) is considered the canine counterpart of Alzheimer's disease (AD). Changes in the retina such as nerve fibre layer (NFL) and ganglion cell complex (GCC) thinning have been observed in Alzheimer's patients. There is also evidence that visual evoked potentials (VEPs) time-to-peaks are delayed in human patients, raising the question if those changes can also be observed in dogs. The aim of this study was to measure NFL and GCC using optical coherence tomography (OCT), as well as record VEPs in young and old dogs to investigate age related changes in those tests, as well as to stablish a correlation to their amyloid beta (Aβ) peptide levels, for in further studies add clinical, behavioural and necropsy information to evaluate the use of those tests in identifying cognitive impairment.

Flash-VEP recordings and OCT scans of the foveal/parafoveal and peripapillary regions were conducted in a group of 12 beagles (6 young, 6 geriatric). An ELISA quantification of Aβ 42 was done with plasma samples from these dogs. No effect of age on Aβ 42 concentration was found in our samples ($p=0.93$) making identification of cognitively impaired individuals based only on Aβ 42 not possible. Age had a significant effect on GCC in the parafoveal and peripapillary regions ($p=0.03$ and $p=0.04$) and on NFL in the parafoveal region ($p=0.02$). Thinning of GCC and NFL in different parafoveal regions was associated with lower Aβ 42 values and thinning of foveal NFL with increased values. Age had a significant effect on most of the late VEP components. Delayed P2 and P4 time-to-peaks were associated with increased and decreased Aβ 42 respectively. We found that GCC thickness was a more straight-forward and robust measurement than the NFL and it has previously been reported to provide further information about synaptic density as it includes the inner plexiform layer and synapses between the ganglion and the bipolar cells. There was a considerable loss of peripapillary measurements for both GCC and NFL thickness and we consider that the distortion caused by vessels in this region in the dog makes it less suitable for measurements of layer thickness. The age-related changes previously described for OCTs and VEPs in dogs were observed also in this study. OCT-imaging and flash-VEP can readily be done in dogs and may provide additional information relevant for assessing of cognitive function in aging dogs. However, a multimodal approach seems to be unavoidable to determine if retinal morphology and functional testing of the visual pathways can be used as markers for CCD.

Keywords: cognitive dysfunction syndrome, dog, optical coherence tomography, OCT, visual evoked potentials, VEP.

Sammanfattning

Det spontant förekommande symtomkomplex som kallas Kognitivt dysfunktionssyndrom (Canine Cognitive Dysfunction=CCD), betraktas som hundens motsvarighet till Alzheimers sjukdom (AS). Förändringar i näthinnan, såsom i nervfiberlagret (NFL) och ”ganglion cell complex” (GCC), har observerats hos Alzheimers patienter. Det är också visat att visuellt framkallade responser (visual evoked potentials, VEP) är fördröjda hos dessa patienter, vilket leder till frågan om dessa förändringar också kan observeras hos hundar. Syftet med denna studie var att mäta NFL och GCC med hjälp av optisk koherentomografi (OCT), samt registrera VEP hos unga och gamla hundar för att undersöka åldersrelaterade förändringar i dessa tester, samt att fastställa en korrelation till deras amyloid beta-peptidnivåer (Aβ), för att, i ytterligare studier tilläga klinisk, beteendemässig och obduktion information för att utvärdera användningen av dessa tester för att identifiera kognitiv nedsättning
Blixt-VEP registreringar och OCT av fovea / parafoveala och peripapillära områden genomfördes på 12 beaglar (6 unga, 6 geriatriska). Med hjälp av ELISA kvantifierades Aβ 42 i plasma. Ingen ålderseffekt sågs på Aβ 42-nivån i våra prover ($p = 0.93$), vilket gjorde att kognitiv nedsättning baserat enbart på denna parameter inte var möjlig på individnivå. Ålder hade en signifikant effekt på GCC i parafoveala och peripapillära områden ($p = 0.03$ och $p = 0.04$) och på NFL i den parafoveala regionen ($p = 0.02$). En förtunning av GCC och NFL i parafovea var associerad med lägre Aβ 42-värden och en förtunning av NFL i fovea med högre värden. Ålder hade en signifikant effekt på nästan alla sena VEP-komponenter. Fördröjda P2- och P4-latenser var förknippade med högre respektive lägre Aβ 42. Vi fann att GCC är ett mer praktiskt och robust mått än NFL och det har tidigare föreslagits att det ger ytterligare information om synaptisk densitet eftersom det också inkluderar det inre plexiforma lagret och synapser mellan ganglioncellerna och de bipolära cellerna. En stor andel av de peripapillära mätningarna av både GCC och NFL kunde inte användas och vi anser att distorsionen som orsakas av blodkärlen i det här området hos hund gör det mindre lämpligt för mätning av tjockleken på näthinnans lager. De åldersrelaterade förändringarna som tidigare beskrivits för OCT och VEP hos hundar sågs också i denna studie. OCT och blixt-VEP fungerar väl på hund och kan bidra med ytterligare information kring kognitiv nedsättning hos åldrande hos hundar. Samtidigt ser vi att det krävs en multimodal ansats för att avgöra om näthinnemorfologi och testning av synbanornas funktion kan användas som markörer för CCD.

Nyckelord: kognitiv dysfunktion syndrom, hund, optisk koherenstomografi, OCT, visual evoked potentials, VEP.

Table of Contents

1. Introduction 1

2. Literature Review 2

2.1. *Canine Cognitive Dysfunction (CCD)* 2

2.1.1. Clinical signs and prevalence 2

2.1.2. Pathophysiology 3

2.1.3. Diagnosis 4

2.1.4. Treatment 5

2.2. *The eye as an Alzheimer's and CCD biomarker* 6

2.2.1. Optical Coherence Tomography (OCT) 6

2.2.2. Visual Evoked Potentials (VEPs) 8

3. Material and Methods 9

3.1. *General Aspects* 9

3.1.1. Animals 9

3.1.2. Physical and ophthalmic examinations 10

3.1.3. Sedation and dilation of pupils during OCTs and VEPs 10

3.2. *Retinal Morphology Assessment: OCT scans* 11

3.2.1. Equipment 11

3.2.2. Procedure and Scans 11

3.2.3. Target regions and measurements 11

3.3. *Post-retinal Pathway Assessment: flash-VEPs* 12

3.3.1. Equipment 12

3.3.2. Electrode placement and VEP testing 12

3.3.3. Measurements 13

3.4. *Laboratory Analysis* 14

3.4.1. Sample collection and handling 14

3.4.2. Aβ 1-42 Enzyme Linked Imunosorbent assay 14

3.5. *Data and Statistical Analysis* 14

4. Results 15

4.1. Aβ Concentration 15

4.2. GCC and NFL measurements for the peripapillary region 15

4.3. GCC and NFL for the foveal region 17

4.4. Flash-VEPs 18

5. Discussion 19

6. Conclusions 24

Popular Scientific Article 25

Acknowledgements 26

References 27

List of Tables

Table 1. Prevalence for CCD by different authors……..…….3
Table 2. Distribution data of the study Beagles…...…….10
Table 3. Mean peripapillary NFL and GCC (µm) and SD (µm). ……..16
Table 4. NFL and GCC foveal and parafoveal global thickness. …….17
Table 5. Flash-VEP components by age group……...18

List of Figures

Figure 1. Retinal layers and structures visualized in the OCT scan of a normal human subject.. 7
Figure 2. Flash-VEP waveforms of 3 Beagles.. 9
Figure 3. OCT image of the canine fovea. 12
Figure 4. Electrode placement for VEP testing. 13
Figure 5. Individual Aβ 42 concentration (pg/mL) and age in years of the 12 dogs…….....15
Figure 6. Percentage of overall peripapillary GCC and NFL measurements affected by vessels at 500 and 1000 µm distance from the ONH. 16

Abbreviations

Aβ	Amyloid beta peptide
AD	Alzheimer's Disease
APP	Amyloid Precursor Protein
BSA	Bovine Serum Albumin
CCD	Canine Cognitive Dysfunction
CSF	Cerebral Spinal Fluid
cSLO	Confocal Scanning Laser Ophthalmoscopy
CT	Computed Tomography
DNMP	Delayed non-matching to position
ELISA	Enzyme Linked Immunosorbent Assay
ERG	Electroretinogram
GCC	Ganglion Cell Complex
GC-IPL	Ganglion cell – Inner Plexiform Layer
HRP	Horse Radish Peroxidase
IM	Intramuscular
ISCEV	International Society for Clinical Electrophysiology of Vision
MAO	Monoamine Oxidase
MCI	Mild Cognitive Impairment
MRI	Magnetic Resonance Imaging
NFT	Neurofibrillary Tangles
NFL	Nerve Fiber Layer
ONH	Optic Nerve Head
PBS	Phosphate Buffered Saline
p-Aβ	Plasmatic Aβ
p-Tau	Phosphorylated Tau
SD-OCT	Spectral Domain Optical Coherence Tomography
VEPs	Visual Evoked Potentials

1. Introduction

Similarly to humans, dogs can present age-related neurodegeneration and associated cognitive impairment with a high degree of individual variability, going from successful agers to those afflicted with pathological cognitive deficits (Vite & Head, 2014; Head, 2013).

Canine cognitive dysfunction (CCD) or canine dementia, is a progressive neurobehavioral syndrome characterized by loss of several cognitive abilities in dogs that has been extensively studied as a natural model for its human counterpart, dementia of Alzheimer's type (Mihevc & Majdic, 2019; Schütt *et al,* 2018; Mazzatenta *et al,* 2017; Head, 2013; Landsberg *et al,* 2012).

Many aspects are shared between CCD and AD, such as decline of several cognitive domains, (Head, 2013; Landsberg *et al,* 2012; Rosado *et al,* 2012a; Rosado *et al,* 2012b; Azkona *et al* 2009), presence of progressive stages of impairment (Madari *et al,* 2015; McKhann *et al,* 2011;) and common neuropathological mechanisms, such as deposition of toxic amyloid beta peptides (Aβ) within the brain parenchyma (Schütt *et al,* 2018; Nesic *et* al, 2016; Colle *et al,* 2000). These peptides can serve as biomarkers detected in the cerebral spinal fluid (CSF) and plasma/serum of affected human and canine patients (Hansson *et al,* 2018; Borghys *et al,* 2016; González-Martinez *et al,* 2011; Head *et al,* 2010).

CCD, however, is also challenging to diagnose, as, besides the difficulty of drawing the line between expected age-related behavioural changes and pathologic ones, aged dogs often present concomitant medical conditions and decreased sensory capability (Landsberg, *et al,* 2012), making the search for objective testing extremely important.

For human patients, it has been suggested that there are retinal changes associated with Alzheimer's disease (AD), such as thinning of ganglion cell related layers, for instance the nerve fiber layer (NFL) and ganglion cell complex (GCC) (Tao *et al,* 2019; Liao *et al,* 2018; Haan *et al,*2017; Hart *et al,* 2017). Those changes can be determined in living patients with new imaging technologies, such as optical coherence tomography (OCT), which allows '*in vivo'* high-resolution visualization and measurement of retinal cellular layers (McLellan & Rasmussen, 2012; Helb *et al,* 2010).

In the same manner, visual evoked potentials (VEPs) have been reported as a diagnostic tool in diagnosing AD, with altered time-to-peaks reported in affected patients (Wyatt-McElvain *et al,* 2018; Tartaglione *et al,* 2012; Kergoat *et al,* 2002).

Hence, it is of interest to investigate if OCTs and VEPs indirectly can provide information about the cognitive function in dogs to further explore interspecies similarities and potentially make discoveries that would be beneficial for both.

The aim of this study was to measure NFL and GCC using OCT technology, as well as VEP responses in dogs of different ages, and relate to their plasmatic Aβ levels to initially investigate their correlations. This project was designed as a pilot for a broader longitudinal study of the progression of observed changes and correlations, adding clinical/behavioural information and ultimately combine these with necropsy findings to assess the usefulness of those tests in identifying cognitive impairment and CCD.

2. Literature Review

2.1. Canine Cognitive Dysfunction (CCD)

2.1.1. Clinical Signs and Prevalence

CCD is characterized by changes in behaviour of senior dogs that are thought to reflect pathological dysfunction of different cognitive domains (Landsberg *et al*, 2012; Rosado *et al*, 2012). The acronym DISHA often is used to describe typical signs reported by the owners, which represents: **D**isorientation, alterations in social **I**nteractions, **S**leep-wake cycle disorders, **H**ouse-soiling and changes in **A**ctivity levels (Schütt *et al*, 2018; Benzal & Rodriguez, 2016; Dewey, 2015; Landsberg *et al*, 2012).

Increased anxiety and phobias, as well as decreased olfaction are also described in CCD, which parallels common signs reported in AD patients, such as agitation and anxious behaviour, and olfactory deficits (Schütt *et al*, 2015; Landsberg *et al*, 2012). The early clinical signs reported in AD patients, deficits in learning and memory, are challenging to identify in dogs, but can be more readily detected in dogs with highly demanding activities (such as service dogs) that can present declined performance (Landsberg *et al*, 2012).

Many epidemiological studies have been made on CCD, which seems to have a prevalence of 14.2% to 22.5% in the canine geriatric population (Salvin *et al*, 2011; Azkona *et al*, 2009; Neilson *et al*, 2001). The syndrome is likely to be under-diagnosed in veterinary medicine, as a large survey with 497 responders (Salvin, *et al*, 2010) demonstrated that only 1.9% of dogs with reported signs of cognitive impairment had a confirmed veterinary diagnosis of CCD.

The prevalence of CCD appears to increase with age (Table 1), and age-related increase in disease severity is also proposed (Salvin, *et al*, 2011; Azkona *et al*, 2009). Regarding sex, females possess more than twice the risk of developing CCD than males (Azkona *et al*, 2009), while it appears that there are no significant differences between dogs of different breeds and sizes when longevity corrections are made (Salvin *et al*, 2011).

***Table 1**. Prevalence for CCD by different authors.*

Reference	Sample size	Method	Age (years)	Prevalence
Salvin *et al* (2010)	497	Survey via online and magazine questionnaires	8-19	14.2%
Askona *et al* (2009)	325	Phone survey	9-11	14.8%
			12-14	29.5%
			15-17	47.6%
Neilson *et al* (2001)	180	Phone survey	11-12	28%
			15-16	68%

2.1.2. Pathophysiology

The pathogenesis of CCD is, as for AD, multifactorial and difficult to unveil. There are several mechanisms implicated in both human and canine counterparts, which might yet to be clarified in terms of causality versus correlation, and that might interact with each other.

The accumulation of toxic Aβ in the form of extracellular plaques and perivascular deposits, as a result of alternative degradation of the transmembrane protein, amyloid precursor protein (APP) is suggested to be the main causative mechanism behind AD and the same is proposed for CCD (Chen *et al*, 2017; Vite & Head, 2014; Landsberg *et al* 2012). In the amyloidogenic pathway processing, APP is split into several differently sized isoforms, which are further cleaved into the main final Aβ forms, 40 (Aβ 40) and 42 (Aβ 42) amino-acids long, which may aggregate into soluble oligomers or dense insoluble fibrils (Chen *et al*,2017; Head *et al*, 2010). Increased Aβ levels in turn, cause neuronal cell death and synaptic failure, with the oligomeric soluble forms being recently implicated as most toxic, binding to cell membranes and many molecules in the extra-cellular space (Chen *et al*, 2017).

The age-related Aβ deposition in the canine brain was suggested to be progressive and region specific by Head e*t al* (2000), who demonstrated that deposition was seen initially and most consistently in the frontal cortex, with an increase in Aβ load and further cortical involvement as age progressed. Osawa *et al* (2016) observed that diffuse Aβ depositions increased in canine brains older than 14 years of age, although the Aβ in the form of senile plaques only increased until this age to then decrease in older dogs. The correlation between brain Aβ load and actual cognitive impairment is also controversial as some authors present a positive correlation (Vite & Head, 2014; Colle *et al*, 2000; Cummings *et al*, 1996), but more recent studies have not come to this conclusion (Osawa *et al*, 2016; Schütt *et al*, 2015) suggesting that Aβ deposition might not be the sole cause of CCD and underlying mechanisms are yet to be discovered. As for example, Osawa *et al* (2016) suggested that an increase in ubiquitin and glial cells might be better markers of CCD pathology than Aβ plaques.

The phosphorylated form of the microtubule associated protein tau (p-Tau) is also described in cognitively impaired dogs (Schmidt *et al*, 2015; Vite & Head, 2014; Landsberg *et al*, 2012), but differently from humans, the structures called neurofibrillary tangles (NFT), the aggregated fibrils of p-Tau, are rarely reported in dogs (Smolek *et al*, 2016; Schmidt *et al*, 2015).

NFT and Aβ are pathological hallmarks of AD (Mihevc & Majdic, 2019) and it is theorised that CCD pathology mimics early stages of AD, due to the presence of p-Tau which could be a pre-tangle development, as well as the Aβ plaque pattern in the dog, which appear less mature then the dense depositions seen in AD. This suggests that CCD never reaches the severity of more advanced stages of AD due to differences in the life-span of dogs and humans (Landsberg *et al*, 2012).

Increased free-radical damage due to losses of compensatory mechanisms with age; neuroinflammation with presence of astrogliosis and microglial activation in proximity to Aβ plaques; neurodegeneration with neuron loss and reduction of brain volume and cortical atrophy, vascular damage, as well as disturbances in serotonergic, noradrenergic and cholinergic neurotransmitter homeostasis (Schütt *et al*, 2018; Benzal & Rodriguez, 2016; Dewey, 2015; Landsberg *et al*, 2012) are all further mechanisms associated with CCD that are far from being fully understood.

2.1.3. Diagnosis

The diagnosis is based on exclusion of other medical and purely behavioural causes for the clinical signs through history taking, physical examination and ancillary tests, such as laboratory analyses (*e.g* complete blood count, blood biochemistry, thyroid hormone levels). Advanced imaging such as computed tomography (CT) or magnetic resonance imaging (MRI) and CSF analysis may be used to rule out systemic and/or structural brain lesions (Dewey, 2015; Landsberg *et al*, 2012). Furthermore, MRI investigations for cortical atrophy and ventriculomegaly, or more objectively interthalamic adhesion thinning, have also been suggested to be possible markers of CCD in the brain (Noh *et al*, 2017; Tapp *et al*, 2004).

Owner-based questionnaires are often used to aid a more detailed assessment and monitoring of behavioural signs and to provide quantitative information through scoring different presentations (Madari *et al*, 2015; Salvin *et al*, 2011; Rofina *et al*, 2006). There are several questionnaires available to aid veterinary professionals. The canine cognitive dysfunction rating scale (CCDR) described by Salvin *et al*, 2011, has been reported to have diagnostic accuracy close to 99% with negative and positive predictive values of 77.8% and 99.3% in diagnosing CCD, respectively. Based on the answers to different questions, such as if they have changed their responses to familiar objects or people, or if they wander restlessly during the day (and how frequently that occurs), dogs can be scored into "unaffected" or normal, to be "at risk" or present mild cognitive impairment (MCI), or to have CCD (Madari *et al*,2015; Salvin *et al*, 2011). Dogs can then be re-tested periodically to assess disease progression. However, the use of questionnaires as stand-alone diagnostic tool, relies on owner assessment and

sometimes on quantification certain behaviours and differentiation between abnormal behaviour caused by CCD and other diseases affecting behaviour and performance.

In the laboratory environment, standardized neuropsychological tests such as the delayed non-matching to position (DNMP) can objectively assess different domains including memory and spatial learning. The test assesses short-term visuospatial memory by evaluating the dogs' performance on identifying the location of an object previously placed in the opposite side of the testing settings (Adams *et al,* 2000). Subtle changes, seen as decline in test performance can be observed as early as 6 to 7 years of age (Landsberg *et al,* 2012; Adams *et al,* 2000). These tests are, however, laborious and require previous training of both the examiner and the tested subject, not being practical for clinical routine.

Moreover, the quantification of levels of Aβ in blood and CSF has been suggested to be linked with Aβ load in the brain and cognitive status in both humans and dogs (Borghys *et al,* 2017; Schütt *et al,* 2015; González-Martinez *et al,* 2011; Head *et al,* 2010), with possible diagnostic usefulness. González-Martinez *et al* (2011) and Schütt *et al* (2015) have described increased p-Aβ concentration in cognitively impaired dogs compared to in unaffected dogs within the same age group, but do comment that these tests have limitations due to high inter-individual variation and dynamic mechanisms related to Aβ production and deposition in the brain.

2.1.4. Treatment

No specific therapy is described for CCD, only options to slow progression and to maintain quality of life.

Environmental enrichment and provision of mental stimuli are pillars for managing CCD and can help affected patients to improve functionality and slow progression of cognitive decline, as well as decrease stress and provide a better animal welfare (Milgram *et al,* 2003; Landsberg *et al,* 2012; Landsberg, 2005).

Evidence exists that dietary management with addition of antioxidants, such as vitamin E and C, essential fatty acids, mitochondrial cofactors and more recently, medium-chain triglycerides (MCT), might improve cognitive function (Dewey, 2015; Landsberg, 2005) with some commercial diets for "mental health support" being available in the market.

For pharmacological treatment, different drugs have been used for improving clinical signs and slow the progression of CCD, with different levels of efficacy. Selegiline, an inhibitor of monoamine oxidase B (MAOB) used in patients with Parkinson's disease to restore dopamine levels in the cortex and hippocampus, has been shown to deaccelerate disease progression and improve overall clinical signs with variable degree of response in CCD patients (Dewey, 2015; Landsberg, 2005). Other drugs, such as propentofylline, a xanthine derivative improving brain blood flow (registered in Sweden under the trade name Canergy Vet. (Virbac Danmark A/S Filial Sverige, Kolding, Denmark)) with the indications "for improving fatigue, lethargy and general cognition in dogs", and the acetylcholinesterase inhibitor phenserine (unavailable commercially for dogs so far) are also mentioned as possibilities. (Dewey, 2015).

Also, recently the use of Aβ immunotherapy for AD has been investigated in dogs. In the study conducted by Davis *et al* (2017) it was observed that the combination of injections of anti-fibrillar vaccine Aβ1-42 and environmental enrichment resulted in maintenance of cognitive abilities and reduced Aβ load in the brain when compared to control beagles.

2.2. The Eye as an Alzheimer's and CCD Biomarker

The retina is considered an accessible part of the central nervous system and a trend of investigations using retinal imaging in the search for morphometric biomarkers for AD has emerged (Liao *et al,* 2018; Hart *et al,* 2016). Retinal morphometry has yet not been studied in CCD dogs.

Impaired visuospatial ability has been associated with AD, and it has been demonstrated that cortical activity evaluated by VEPs is impaired in those patients (Tartaglione *et al,* 2012; Wright *et al,* 1986). Changes in VEP responses have also been observed in dogs with CCD (Hamnilrat *et al,* 2015).

2.2.1. Optical Coherence Tomography (OCT)

Optical coherence tomography is a relatively novel imaging technique based on the principle of low coherence interferometry, which obtains images of biological tissues using their different light scattering/reflection patterns (McLellan & Rasmussen, 2012). OCT has revolutionized clinical ophthalmology with the non-invasive acquisition of very detailed cross-section images of the retina and its different layers (Figure 1), allowing precise measurements of its light-microscopic features (McLellan & Rasmussen, 2012; Helb et al, 2010).

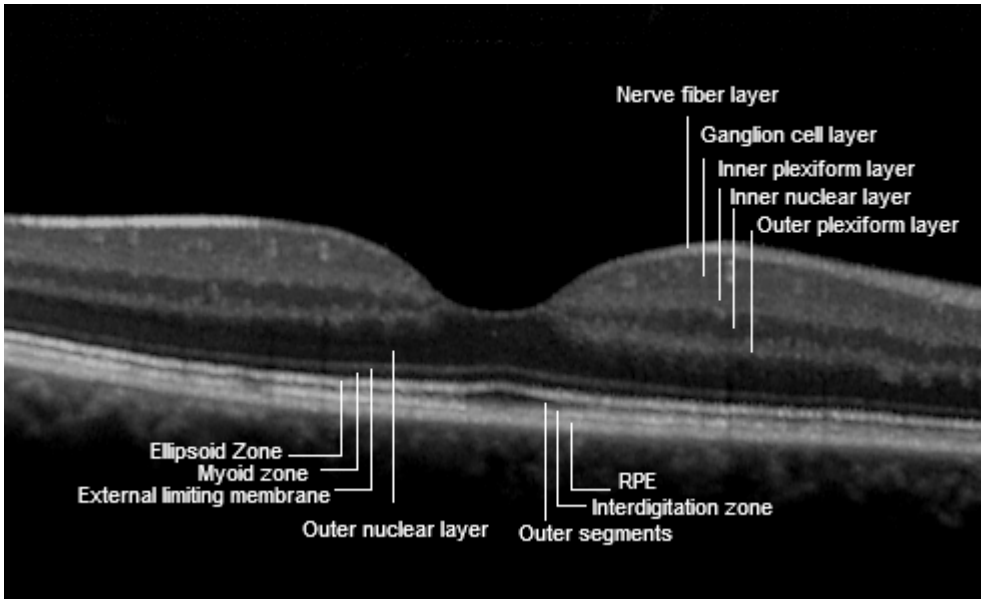


Figure 1. *Retinal layers and structures visualized in the OCT scan of a normal human subject. Landmarks based on: Staurenghi, G., et al.,* ***Proposed lexicon for anatomic landmarks in normal posterior segment spectral-domain optical coherence tomography: the IN* OCT consensus.*** *Ophthalmology, 2014. 121(8): p. 1572-8.*

Regarding AD, it has been demonstrated that Aβ and Tau are also found in the retinas of AD-patients and the damaging effects of their deposition can lead to ganglion cell death and thinning of the retinal NFL, and that OCT imaging has been successful in identifying those structural changes (Liao *et al,* 2018; Hart *et al,* 2016).

Other morphological changes in the retina, such as thinning of the total retinal thickness and decrease in macular volume have also been positively correlated with AD and reflect disease severity (Liao *et al,* 2018). A direct correlation between OCT macular retinal thickness and brain volume is described by Tao *et al* (2019) in MCI patients, with a stronger association between the perifoveal inner retinal thickness and the volume of hippocampal and entorhinal cortices, supporting that retinal and brain AD pathology are linked.

Thinning of the retinal NFL surrounding the ONH (peripapillary region) is one of the highlighted and most studied OCT findings in AD retinas. However, there are conflicting results and the predictive value of this measurement has been questioned (Sanchéz *et al,* 2018; Haan *et al,*2017). It has recently been suggested that the thickness of the GC-IPL (ganglion cell and inner plexiform layer) or the GCC (NFL, ganglion cell layer and inner plexiform layer), in the perifoveal/macular region are more sensible methods for detecting AD and MCI (Tao *et al,* 2019; Marziani *et al, 2013*).

In veterinary patients, OCT imaging has also been conducted (Ofri & Ekesten, 2019; Hernandez Merino et al, 2011) and baseline values and specific characteristics of the normal and diseased canine retina are recently being explored (Graham *et al,* 2019; Braga-Sá *et al,* 2018). Ofri & Ekesten (2019) described measurements at eccentricities from 1 to 6 mm from the optic nerve head (ONH) for total retinal thickness, outer retinal thickness and NFL in

female beagles, and report that thickness varies between different retinal meridians and that retinal thinning is significant from puppyhood to adult age, but not later in life occurs with age.

Beltran *et al* (2014), also used OCT scans to identify a cone dense, fovea-like structure in the canine eye (which was previously thought to be inexistent), that is preferentially affected by mutations (*BEST-1 and RPGR* genes) causing forms of macular degenerations in humans, but is also affected early in dogs with a mutation in ABCA4 causing a canine form of Stargardt disease (Mäkeläinen *et al*, 2019). Hence, this retinal area, the fovea plana, is considered the canine counterpart to the primate fovea in the centre of the macular area.

2.2.2. Visual Evoked Potentials (VEPs)

Visual evoked potentials are electrical potentials recorded in response to a light stimulus, which reflect the activity of the different structures involved in the visual pathway from the retina to the visual cortex (Kimotsuki *et al*, 2005). VEPs can be recorded non-invasively from scalp electrodes and the responses are used to assess the integrity of the visual pathway structures and detect mainly post-retinal lesions (Odom *et al*, 2016).

Guidelines for VEP testing in humans are available and constantly updated by the International Society for Clinical Electrophysiology of Vision (ISCEV). Three types of VEP protocols are described by the ISCEV: pattern reversal, pattern onset/offset, and flash, with pattern reversal usually being preferred for human patients due to more consistent responses, and flash VEPs used when poor cooperation is expected. Six wavelets are described for flash-VEPs in humans, starting with a negative component (N1) (Odom *et al*, 2016).

As previously mentioned, visuospatial impairments are associated with AD, which are chiefly attributed to damage to the primary visual cortex and association areas. VEP findings in dementia patients was first described by Wright *et al* (1986), who observed an increased P2 time-to-peak in flash-VEP and normal pattern VEPs in patients with dementia, and that this combination was shown to be more specific than CT and EEG in diagnosing the disease.

VEPs have been described in many animal species besides humans. For dogs, five flash-VEP components have been described (P1, N1, P2, N2, P3), resembling a "M-shaped" waveform with P2 being the most prominent (Figure 2). A number of authors have reported normal time-to-peaks and amplitudes in dogs, but preparation of patients, protocols and criteria for identification and nomenclature of the different peaks and troughs vary between studies (Torres &Tovar, 2016; Kimotsuki *et al*, 2006; Kimotsuki *et al*, 2005; Strain *et al*, 1990).

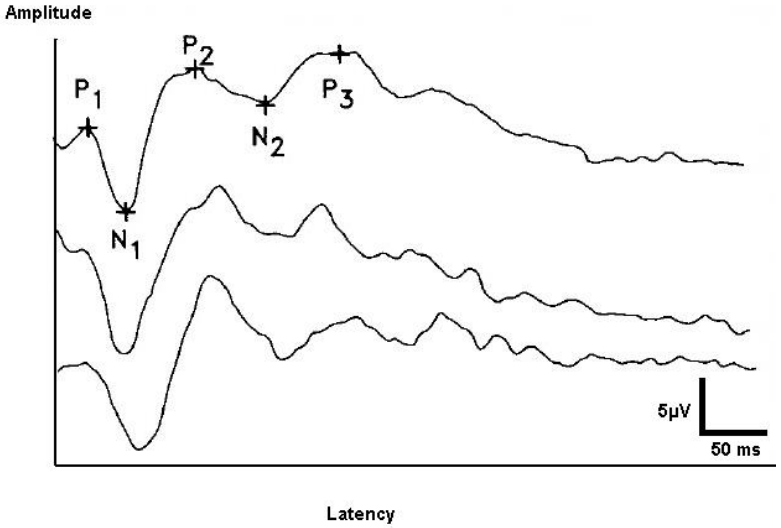


Figure 2. *Flash-VEP waveforms of 3 Beagles. Modified from: Strain et al, 1990.* ***Visual Evoked Potentials in the clinically normal dog.*** *Journal of veterinary internal medicine, 4(4), pp.222-225.*

The generators of each component of the flash VEP has been investigated trough topographic mapping by Kimotsuki *et al* (2005). In this study, P1 and N1, P2, N2 were suggested to reflect activity in the retina, from the retina to brain stem, and from brain stem to visual cortex, respectively.

A few studies have reported a correlation between canine dementia and delayed flash-VEP time-to-peaks. Significantly delayed P2 and P3 time-to-peaks have been associated with cognitive impairment in a study of 28 Pomeranian dogs recently (Hamnilrat *et al,* 2015). Kimotsuki *et al* (2006) reported that longer P2, N2, P3 time-to-peaks, as well as decreased peak-to-peak amplitudes for P2-N2 and N2-P3 were associated with increased age, and even though the cognitive status of the dogs in this study was not assessed, the authors also speculate the potential value of flash-VEP testing for dementia in dogs.

3. Material and Methods

3.1. General Aspects

3.1.1. Animals

This study was conducted at the Swedish University of Agricultural Sciences (SLU), Uppsala, Sweden, and the study dogs were 12 female Beagles (11 intact, 1 spayed) from the University's

colony of laboratory animals. Six dogs from 9 years of age were categorized into the 'Old group' and six dogs below 3 years of age were categorized into the 'Young group', which also served as the control group. Furthermore, the old dogs were then subdivided into 2 groups, one with four dogs with <11 years and one group with two dogs ≥11 years (Table 2).

The use of animals in this study was approved by the regional animal ethics committee of the district court in Uppsala, Sweden (Uppsala Djurförsöksetiska nämnd dnr 5.8.18-15533/2018), in accordance with the EU directive for protection of animals used for scientific research (EU63/2010) and was conducted in accordance with the Statement for the Use of Animals in Ophthalmic and Visual Research published by the Association for Research in Vision and Ophthalmology.

Table 2. *Distribution data of the study Beagles.*

Age group	**Young (n=6)**	**Old (n=6)**	
		Old < 11 years (n=4)	**Old ≥11 years (n=2)**
Weight (mean ± SD)	13.3 ± 0.4	12.8 ± 1.3	11 ± 0.5
Age	2 years (siblings)	9 years (n=3); 10 years (n=1)	12 years
Neutering status	6 intact	3 intact, 1 spayed	2 intact

3.1.2. Physical and Ophthalmic Examinations

The dogs were submitted to physical and ophthalmic exams prior to the OCTs and VEPs and had blood collected for the measurement of p-Aβ. The care-taker of the dogs was asked about the behaviour of individual dogs (especially relating to social interactions) to obtain a general overview of their cognitive status, as several questions in the standard owner-based questionnaires were not applicable for laboratory animals. The ophthalmic examination included assessment of ocular reflexes (pupillary and dazzle reflexes) and menace responses, rebound tonometry (Tonovet, Icare Finland Oy, Vanda, Finland), as well as slit lamp biomicroscopy and indirect ophthalmoscopy after dilation with tropicamide (Tropikamid, 0.5%, Bausch & Lomb Nordic AB, Stockholm, Sweden).

3.1.3. Sedation and Dilation of Pupils During OCTs and VEPs

OCTs and flash-VEPs were performed with at least 1 week apart in all dogs. Mydriatic eye drops (Tropikamid, 0.5%, Bausch & Lomb Nordic AB, Stockholm, Sweden) were instilled at least 30 minutes prior to each test. For OCTs, a combination of medetomidine 0.015-0.04 mg/kg IM (Sedator Vet, 1mg/ml, Dechra Veterinary products AB, Upplands Väsby, Sweden), butorphanol 0.2-0.4 mg/kg IM (Dolorex Vet, 10mg/kg, Intervet AB, Stockholm, Sweden) and ketamine 0.6-1.5 mg/kg IM (Ketaminol Vet, Intervet AB, Stockholm Sweden) was used for sedation, with the low-end doses preferred for the old group. This protocol was developed by

the anaesthetic team to enhance OCT examination and will be presented in a separate report. For the flash VEPs, dogs were sedated with medetomidine and butorphanol (0.005 and 0.05 mg/kg, respectively). After the testing, sedation was reversed with atipemazole (Atipam Vet, 5mg/ml, Dechra Veterinary Products AB, Upplands Väsby, Sweden).

3.2. Retinal Morphology Assessment: OCT scans

3.2.1. Equipment

For the retinal scans, a spectral-domain Heidelberg Spectralis HRT+OCT (Heidelberg Engineering GmbH, Heidelberg, Germany) was used to obtain both images of the posterior segment (cSLO) and cross-sections (OCT). The device was connected to a desktop computer with the Heidelberg Eye explorer software (Heyex, Heidelberg Engineering, Version 1.9.14.0, 2016), for image acquisition and assessment. Both standard and wide-field lenses (30º and 55º) were used.

3.2.2. Procedure and Scans

After the desired level of sedation was achieved, the dogs were placed in sternal recumbency at an examination table with their heads set on a chin rest facing forward. The eyes were kept open either manually or using Barraquer lid specula (after topical anaesthesia with Oxybuprocaine 0.4% (Bausch & Lomb Nordic AB, Stockholm, Sweden) and were kept moist with artificial tears (Bion Tears, Alcon Nordic A/S, Copenhagen, Denmark) throughout the exam. Monitoring of vital parameters and sedation depth was performed using a multiparameter monitor (B40 patient monitor, GE Medical systems, Freiburg, Germany).

Firstly, infrared and fundus autofluorescence cSLO images were obtained from both eyes to be used for overview of the fundus and guidance in identification of the area centralis and fovea plana. Secondly, each eye was scanned in the peripapillary and foveal regions (target regions for this project), using radial scans centred at the ONH and volume scans respectively, and images were saved for later processing.

3.2.3. Target Regions and Measurements

For the fovea, we scanned the visual streak area approximately 4.5-5 mm temporal the ONH (Beltran *et al*, 2014) with 60 µm between each b-scan (cross-section). The fovea was identified by a tiny indentation of the innermost retinal layers, and an elevation of the external limiting membrane, ellipsoid and interdigitation zones at its centre (Figure 3). NFL and GCC were measured at the centre and at 180 µm to the temporal, superior, nasal and inferior aspect of the fovea (all four measurements representing the parafoveal region).

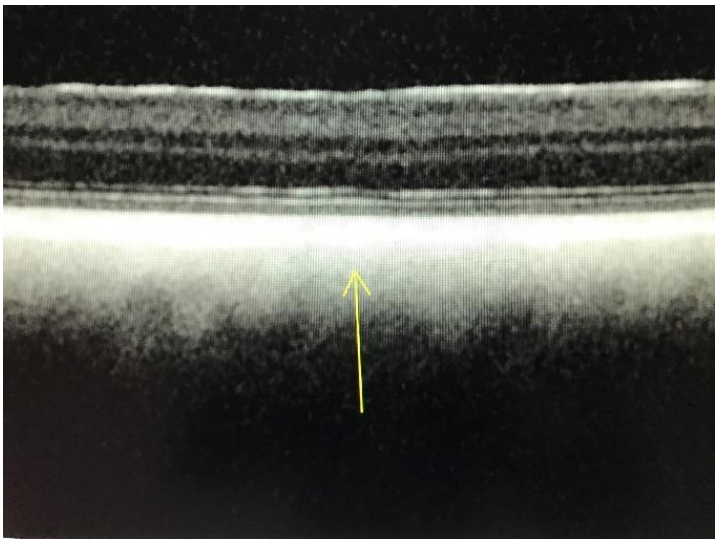


Figure 3. *OCT image of the canine fovea.*

For the peripapillary area, 12 radial b-scans were obtained around the ONH starting at 12 o'clock. Then the ONH would be delimited visually at the cSLO images and measurements of NFL and GCC layers were made for each scan at 500 and 1000 µm from the myelin border outlining ONH. The radial scans were subdivided into groups (dorsal, medial, lateral and ventral) based on their clock-hour-position. In some images the eye globe was tilted, and landmarks in the cSLO images, such as the retinal veins, visual streak, choroidal vessels, had to be used to apply the clock reference and assign the scans to the proper meridian.

3.3. Post-retinal Pathway Assessment: flash-VEPs

3.3.1. Equipment

For the flash-VEPs an ERG/VEP system (PowerLab, AD instruments Inc, Sydney, Australia) was used with a 4-channel amplifier (Quad BioAmp, AD instruments Inc, Sydney, Australia). The background light and flashes were produced in a custom-built full-field stimulator fitted with a xenon strobe (Grass PS33+, Astro-Med Inc., Grass Instrument Division, West Warwick, RI, USA). The recordings were controlled and analysed using a special software (LabChart 8.1.17, AD instruments Inc, Sydney, Australia) in a laptop computer connected to the system.

3.3.2. Electrode placement and VEP testing

After the desired level of sedation, disposable subdermal single needle electrodes (Technomed Europe, Limburg, The Netherlands) were placed based on the international 10/20 system (Odom, *et al*, 2016) described for humans (Figure 4), with 3 active VEP electrodes being placed (Oz, O1 and O2), at 30% of the distance measured from the nuchal crest to the junction between nasal canthi and 1.5 cm to the left and right to midline, respectively. The ground electrode was placed in the midline at the vertex and the reference electrode was placed at the forehead (Fz),

30% caudal from the nasal canthi. For simultaneous ERG recordings from the stimulated eye, disposable corneal jet electrodes (ERG-jet, Universo Plastique AS, Grenchen, Switzerland) were used. Electrode impedance was kept below 5 kΩ.

Figure 4. *Electrode placement for VEP testing. Notice that the left eye is patched to allow unilateral stimulation.*

Light-adapted flash-VEPs were performed in the Ganzfeld stimulator with a steady 25 cd/m² background light and flashes with a luminance over time of 3.0 cd/m²/s presented at 1.09 Hz. The room lights were turned off to avoid interference from ambient light

The dog was positioned in sternal recumbency with their eyes just inside the opening of the Ganzfeld dome. Monocular stimulation was conducted while the fellow eye was covered with a dark, opaque plastic cover kept in place by tape. At least 2 VEPs were recorded from each eye and 100 responses were averaged for each VEP. Recordings from both eyes, one at a time were obtained.

3.3.3. Measurements

ERG a- and b-waves were identified, and amplitudes and times-to-peaks measured to ensure that the retina responded to the light stimulus. Then VEP tracings from all dogs were compared to identify peaks and troughs previously reported (Torres & Tovar, 2016; Kimotsuki *et al*, 2005; Strain *et al*,1990; Sims *et al*, 1989; Bischel *et al,1988*), as well as late responses that have not been previously described.

Reproducible peaks and troughs were named after polarity and order of appearance starting with P1. Time-to-peaks and amplitudes were measured from stimulus onset and peak-to-peak, respectively, essentially in accordance with the ISCEV guidelines and previous canine studies (Odom *et al*, 2016; Torres & Tovar 2016; Kimotsuki *et al* 2005). There was a total of 12 more or less frequently observed wavelets, 6 positive and 6 negative.

3.4. Laboratory Analysis

3.4.1. Sample Collection and Handling

Blood samples (approximately 12 ml of whole blood) were collected from fasting dogs in the morning, from the cephalic veins into collection tubes containing EDTA (Vacutainer, Becton Dickinson AB, Stockholm, Sweden). The tubes were immediately centrifuged (2.000 x g for 15 minutes at RT), and the separated plasma aliquoted into 1 ml cryotubes (Nunc cryotubes, VWR International AB, Stockholm, Sweden). The tubes were frozen at -80ºC initially.

3.4.2. Aβ 1-42 Enzyme Linked Imunosorbent assay

A sandwich ELISA kit for canine amyloid beta 1-42 peptide (c2ABETA1-42-ELISA, FIVEphoton Biochemicals Inc, San Diego, USA) with 96 wells was used. The sandwich kit contained wells coated with anti-canine amyloid beta 42 capture antibodies and biotin labelled detection antibodies.

The ELISA was performed according to manufacturer's instructions. A preliminary single-strip test was done to observe if there were measurable signals in the chosen samples or if a dilution would be required for the values to fall in the assay range. After reading the preliminary test results, the testing proceeded a second time, now with the entire plate and samples from all 12 dogs. The plate was read at 450 nm to determine the optical density values (absorbance) and Aβ 42 concentration. The samples were assessed in duplicate to account for pipetting errors and to obtain data for CV values and for statistical validation.

3.5. Data and Statistical Analysis

For analysing the data, JMP trial version 15 (SAS Institute Inc, Cary, NC USA) was used. Student's t-test or Wilcoxon signed rank test (depending on distribution of the data determined by the Anderson-Darling test of normality) were used to assess differences between recordings and electrode placement for the flash-VEPs, between quadrants in the peripapillary OCTs, and for differences between eyes from both tests. Linear regression was used to determine the effect of age in GCC/NFL layers for the foveal and peripapillary OCTs and flash-VEPs time-to-peaks/amplitudes, as well as to fit a model for CCD prediction using stepwise approach for both tests separately. The suggested models that were significant and had the greatest adjusted coefficients of correlation (R^2) were chosen. Results were considered significant if $p < 0.05$.

4. Results

In the Old group, the two 12-year-old dogs showed altered behaviour and did not respond to previously learned commands. Two dogs (10 and 12 years of age) had altered ophthalmoscopic appearance. The first had 2 focal intraretinal, petechial haemorrhages in the superior quadrant and the latter had more extensive lesions with retinal oedema (markedly on the temporal region) and more focal intraretinal haemorrhages. On OCT, the latter 12 year-old dog had a considerably thicker retina than any other dog in the foveal/parafoveal measurements, and therefore only the fellow eye (that was considered ophthalmoscopically normal) was used for further analysis in this region. Besides this, there were no statistically significant differences between left and right eyes on the OCT, and therefore all eyes were used.

4.1. Aβ Concentration

When analysing the ELISA plate, a correlation coefficient (R^2) of 0.945 was obtained for the calibrators' concentrations and the average coefficients of variation (CV %) was 3.66 %.

There was no obvious correlation between Aβ 42-values and age(p=0.93), although one dog in the Old group had the highest concentration (Figure 5).

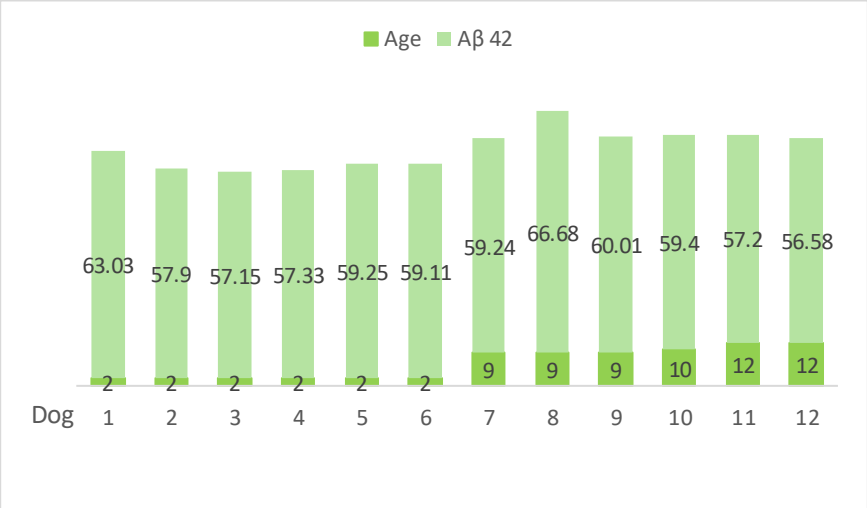


Figure 5. *Individual Aβ 42 concentration (pg/mL) and age in years of the 12 dogs. A rather low variation between all dogs tested can be noticed.*

4.2. GCC and NFL Measurements for the Peripapillary Region

A large fraction of the peripapillary data points at 500 µm distance from the ONH, on average 44%, could not be measured from the OCTs due to presence of vessels distorting the layers to be measured (Figure 6). At 1000 µm from the ONH more data points showing either NFL or GCC thickness could be measured, but still 36% of the images were marred by traversing retinal blood vessels.

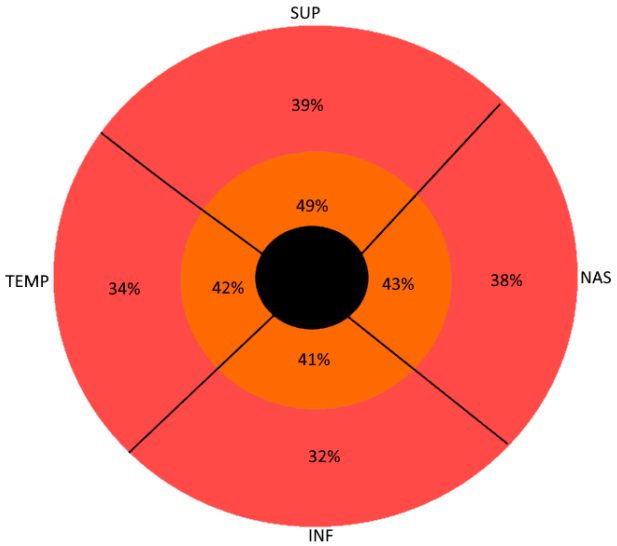


Figure 6. *Percentage of overall peripapillary GCC and NFL measurements affected by vessels at 500 (orange) and 1000 µm (red) distance from the ONH. SUP= Superior, NAS= nasal, TEM= Temporal, INF= Inferior.*

The peripapillary measurements at 1000 µm for NFL and GCC that were clear of vessels were chosen for further analysis and are described in Table 3 by retinal meridians and age groups. The peripapillary GCC measurements have a greater variation in the superior and nasal quadrants. Both parameters seem to be thicker on the superior and thinner in the inferior quadrant.

Table 3. *Mean peripapillary NFL and GCC (µm) and SD (µm). Both parameters most often tapered with increasing age in all locations.*

	NFL	GCC
SUPERIOR		
Young	75 ± 38	109 ± 36.8
Old <11 y	62.6 ±18.2	94.2 ± 16.5
Old ≥ 11 y	54.3 ± 20.4	89.6 ± 23.2
NASAL		
Young	66.3 ± 27.9	97.4 ± 31.1
Old <11 y	59 ± 25.6	91.9 ± 27.3
Old ≥ 11 y	54.6 ± 21.6	85.8± 18.4
TEMPORAL		
Young	65.3 ± 16.6	97.9 ± 16.7
Old <11 y	52.8 ± 16.2	80 ± 14.5
Old ≥ 11 y	57 ± 11	85.2 ± 10.9
INFERIOR		
Young	46.3 ±18.7	74.8 ± 18.8
Old <11 y	39.7 ± 13.1	64.2 ± 12.7
Old ≥ 11 y	39.7 ± 10.1	59.9 ± 6.2

Obs.: mean thickness ±SD at 1000 µm from the ONH.

Age had a significant effect on the overall peripapillary GCC (p=0.049). This effect, however, was not significant for NFL thickness (p=0.056). For the different quadrants, significant differences were only seen between the superior and inferior NFL and GCC measurements (p=0.0001 and p<0.0001, respectively). We also searched for a correlation between peripapillary GCC and NFL and Aβ 42 concentration, but here we could not find a model that was significantly different from a horizontal line.

4.3. GCC and NFL for the foveal region

Differences between age groups in the foveal and parafoveal measurements are demonstrated in Table 4, where the measurements at the centre of the fovea are thinner than those in the surrounding quadrants and that is a progressive decrease between age groups for the parafoveal thickness.

Table 4. *NFL and GCC foveal and parafoveal global thickness. The global parafoveal measurement is the average of the measurements superiorly, nasally, temporally and inferiorly at a distance of 180 µm from the fovea.*

Age group	**Mean thickness (µm) ±SD**	
	GCC global parafoveal	**GCC foveal**
Young	69.1 ± 4.2	64.7 ± 4.2
Old <11 y	60 ± 6.5	58.5 ± 6.4
Old ≥ 11 y	58.5 ± 6.8	59 ± 4.2
	NFL global parafoveal	**NFL foveal**
Young	13.2 ± 2.7	10.3 ± 2.5
Old <11 y	11.2 ± 2.3	8.4 ± 2.9
Old ≥ 11 y	10.1 ± 2.4	9.0 ± 1.4

A significant effect of age was seen for GCC (p=0.003) parafoveal thickness, with thinner measurements associated with old age. At the centre of the fovea the difference was not significant (p=0.07). Old age also resulted in thinner parafoveal NFL (p=0.02), whereas the thinning of the NFL was not statistically significant in the central fovea (p=0.08).

We were also interested in which OCT parameters that best predicted the p-Aβ 42 level. Using the Stepwise regression procedure, the coefficient of correlation for a model including GCC parafoveal temporal, inferior and foveal measurements reached R^2=0.68, p<0.02:

$$\text{p-A}\beta\ 42 = 71.71 + 0.59 \times \text{pfGCC}_{\text{temp}} - 0.33 \times \text{pfGCC}_{\text{inf}} - 0.46 \times \text{fovGCC} + \varepsilon$$

In this model, the parafoveal temporal GCC had a significant effect (p=0.01) where a decrease of 10 µm in GCC thickness was associated with a decrease of 6 pg/mL in p-Aβ values. The

same approach for explaining the variation of Aβ 42 using NFL thickness data showed that a combination of parafoveal nasal and foveal measurements gave a R^2=0.55, p<0.02:

$$p\text{-}A\beta\ 42 = 59.71 + 0.86 \times pfNFL_{nas} - 1.18 \times fovNFL + \varepsilon$$

Here, a significant thinning of nasal parafoveal NFL was associated with decreased p-Aβ levels (p=0.02), in that case a decrease of 5 µm in NFL would result in a 4.3 pg/mL decrease in p-Aβ. However, in the same model, increased Aβ levels were seen with the thinning of the foveal NFL (p=0.01), with the same magnitude of decrease causing an increase of 5.5 pg/mL in p-Aβ.

4.4. Flash-VEPs

There was a total of 12 more or less frequently observed wavelets, 6 positive and 6 negative. No significant differences were seen between ipsilateral and contralateral recordings. Because most of the axons from the retinal ganglion cells decussate at the optic chiasm in the dog, contralateral recordings were chosen for further analysis in this thesis. The VEP components P1, N1, P2, N2, P3, N3 and P4 were seen in all dogs. P5 was seen in all dogs except from one in the Old <11 y group. N5 was seen in all dogs except 2 dogs of the Old <11 y group and one dog of the ≥ 11y group and P6 was missing in 3 dogs of the Old <11 y group. In table 5, the mean peak-times and amplitudes for the 6 VEP components are shown, P2 was the most prominent peak, followed by N2. A considerable difference between time-to-peaks from young and old groups can be noted.

Table 5. *Flash-VEP components by age group.*

Time-to-peak (ms)											
	P1	**N1**	**P2**	**N2**	**P3**	**N3**	**P4**	**N4**	**P5**	**N5**	**P6**
Young	12.2 ±0.5	26.2 ±2	67.2 ±8.8	119.3 ±11.9	146.6 ±17.5	188.3 ±24.5	230.3 ±26.1	278.6 ±24.1	313.5 ±33.3	362 ±18.5	424.5 ±14
Old <11 y	12.9 ±1	24.7 ±2.5	86.7 ±9.5	134.4 ±18.3	164.7 ±25.1	214.7 ±32	254.4 ±37.2	321.7 ±21.8	356.1 ±13.2	400 ±9	433.2 ±18.7
Old ≥11y	19.8 ±14.6	31.5 ±14.4	84.7 ±4	160.1 ±6.1	198.7 ±4.8	248.5 ±12	298.1 ±16.7	347.3 ±23.7	392.7 ±35.2	417 ±0.5	450.2 ±6.5

Amplitude (µV)											
	P1	**N1**	**P2**	**N2**	**P3**	**N3**	**P4**	**N4**	**P5**	**N5**	**P6**
Young	1.3 ±0.1	5.7 ±2	11.6 ±3.5	7.7 ±2.6	1.9 ±0.6	3.4 ±1.2	4.7 ±2.5	3.9 ±1.6	2.9 ±1	2.9 ±1.1	3.8 ±1.2
Old <11 y	1.6 ±0.4	4.4 ±2.3	18.2 ±5.2	10.4 ±3.9	2.5 ±1.3	6 ±0.9	2.5 ±1.3	3.2 ±1.3	2.8 ±1.3	3.2 ±1.9	2.7 ±0.7
Old ≥11y	1.4 ±0.8	1.4 ±0.7	12.2 ±3.5	10 ±1.3	1.9 ±1.1	4 ±1.4	2.5 ±0.4	1.5 ±0.7	3.3 ±2.5	4.9 ±0.2	1.6 ±0.4

Age had a significant effect on the VEP time-to-peaks for P2 (p=0.02), N2 (p=0.01), P3 (p=0.01), N3 (p=0.01), N4 (p=0.001), P5 (p=0.006), N5 (p=0.006), with longer time-to-peaks seen in older age. Age had a significant effect on the negative N3 amplitude (p=0.02) with higher values associated with old age, and in the positive P4 (p=0.04) and P6 (p=0.01) with decreased amplitudes in older dogs

We also fitted linear models to see if we could correlate VEP time-to-peaks to the amount of p-Aβ 42. The combination of VEP time-to-peaks that yielded the highest adjusted R^2 in a model significantly deviating from a horizontal line, was a model including P2 and P4 time-to-peaks with R^2=0.76 (p<0.0018). In this model, the parameter estimates for P2 and P4 both had significant effects (p=0.0045 and p=0.0007, respectively):

$$\text{p-A}\beta\ 42 = 65.78 + 0.15 \times P2_{lat} - 0.07\ P4_{lat} + \varepsilon$$

For P2, an increase of 20 ms in time-to-peak would mean an increase of 3 pg/mL of Aβ, and for P4 the same increase causes a decrease of 1.4 pg/mL in Aβ. Amplitudes had a higher variation and we couldn't obtain a significant model correlating them to p-Aβ 42.

5. Discussion

We have performed a pilot study on p-Aβ 42 concentrations in young and old female Beagles and searched for a relation between p-Aβ 42 and morphometric data from the retina, as well as data reflecting visual cortical function.

Ideally, we would have compared data from dogs with verified cognitive dysfunction and no other diseases to age-matched, fully healthy controls of the same breed, but such a material is extremely difficult to find in aged canine populations. However, CCD *per se* is a challenging diagnosis to make. In the old group, the two 12-year-old dogs showed altered behaviour and did not respond to previously learned commands, which was considered to be related to hearing problems by their care-taker. Landsberg *et al* (2012) discussed that sensory deficits, such as hearing and visual impairment, common findings in aged dogs, may cause behavioural changes, making it difficult to distinguish if they are the cause of seen behaviours or confounders/aggravators of cognitive deficits. Based on the lack of gross behavioural signs, we therefore assumed that in our old population, if any, only dogs with subtle signs or mild cognitive impairment would be present.

We decided to analyse p-Aβ 42, which has been reported to be linked to CCD and also be a marker of CCD progression (González-Martinez *et al*, 2011). We found no statistically significant correlations between p-Aβ 42 concentration and age. One concern was that we observed very little variation between individual dogs compared to other researchers (Schütt

et al, 2015; González-Martinez *et al*, 2011) and our results were close to the background level. We can think of three factors causing the low levels and low variation: the facts that half of the dogs were siblings, problems with the ELISA kit itself, and the stability of the p-Aβ 42 during preparation of samples and storage. Plasmatic amyloid peptides are quite sensitive to storage and decrease in concentration when kept in room temperature or refrigerated for longer periods (Rózga *et al* 2019). Our samples were kept frozen for approximately 1 month, which is longer than the time period for frozen samples studied by Rózga *et al*. However, we find it unlikely that p-Aβ 42 would degrade rapidly in -80° C, so the cause of the low concentrations needs to be sought elsewhere in the chain of events from sampling to analysis, or in the possibility of genetic factors causing less variability in our samples.

The level of p-Aβ 42 being unaffected by age does not match previous reports that plasma/CSF Aβ-peptides decrease with normal aging (González-Martinez *et al* 2011; Head *et al*, 2010), as there is no increased production of Aβ to overcome the age-related deposition of these peptides in the brain parenchyma (González-Martinez *et al* 2011). Although the lack of a significant decrease with age may be attributed to the relatively small sample size in our study, the results warrant further validation of the ELISA-kits used.

In our study, a 9-year-old beagle (in the Old group) had the highest p-Aβ 42 value, which could indicate that this dog might have mild cognitive deficits, although the 2 older dogs with behavioural abnormalities in the same group had lower values. González-Martinez *et al* (2011) and Schütt *et al* (2015) reported that p-Aβ concentrations increase in dogs with cognitive impairment when compared to aged, unaffected dogs. While González-Martinez *et al* (2011) observed increased Aβ 42 concentrations in mildly versus severely cognitively impaired, Schütt *et al* (2015) reported the opposite. One explanation to our results could be that the old dogs with abnormal behaviour had developed more advanced cognitive impairment and p-Aβ 42 was lower because of an increased accumulation of the peptide in the brain parenchyma. We also observed overlapping values within age groups, which is in agreement with both these authors. Hence, we agree that a longitudinal approach may be advantageous for studying what tests may be most useful as early biomarkers.

OCTs could readily be performed in the beagles after appropriate sedation and when the eyes were well centred. However, 36% of the peripapillary measurements were discarded due to the presence of vessels distorting the retinal layers at 1000 µm distance from the ONH, in particular in the superior and nasal quadrants and at 500 µm distance more than 40% had to be discarded for the same reason. Not surprisingly, this coincided with a greater variation of the measurements in those quadrants. This is also in agreement with Bemis *et al* (2017) and Ofri & Ekesten (2019), who reported that NFL measurements can be affected by the presence of vessels, especially in the superior region, and this can affect thickness of retinal layers. The retinal blood vessels also follow the axonal distribution and are greater in areas of thicker NFL.

The NFL and GCC were thicker in the superior quadrant and thinner inferiorly. The variation in thickness between quadrants and thickness of the NFL itself agrees with previous studies

performed on a similar material (Ofri & Ekesten, 2019). Hernandez-Merino *et al* (2011) also used intact female Beagles and observed a similar pattern of the peripapillary NFL. However, the NFL in our study seems to be slightly thinner, particularly in the superior quadrant. This may reflect that dogs of different ages were studied, but also that we excluded all OCTs where retinal blood vessels distorted the retinal layering. However, we found it relevant to study inner retinal thickness as there may be certain, more vulnerable regions associated with cognitive dysfunction, as seen in AD where the superior and inferior quadrants are the thinnest (Haan *et al*, 2017),

We found that often in the peripapillary scans, it was difficult to delimit the NFL, the ganglion cell layer and inner plexiform layer. The same problem was reported by Bemis *et al* (2017), who also raises a concern that automated measurements may not segment these layers correctly and erroneously add them together as the NFL. In our study, GCC results were more consistent compared to NFL not only in the peripapillary region, but also in the area centralis, most likely because the NFL is often so thin that it is difficult to position the measurement bars. Therefore, we advocate measuring the GCC as it is more straight-forward and less prone to operator or software errors.

In this study, the correlation between peripapillary OCT data and p-Aβ was poor, regardless of whether NFL or GCC was measured. The usefulness of retinal NFL in AD is controversial. In a large study involving nearly 930 subjects, Sanchéz *et al* (2018) found no significant decrease in peripapillary NFL in patients with probable minor cognitive impairment or probable AD compared to in controls. However, a large meta-analysis has shown that NFL thickness is decreased in MCI and AD patients compared to in healthy controls (Haan *et al*, 2017). Although our data suggest that peripapillary NFL is a poor predictor of p-Aβ 42, it remains an arguable point if it can be of any value as a biomarker for CCD.

OCTs of the area centralis showed a significant effect of age on both GCC and NFL in the parafoveal region, but no significant age effect was seen at the centre of the fovea. In the peripapillary region, only GCC was significantly affected by age, even though the age effect on NFL almost reached statistical significance. This suggests that the thickness of the innermost retinal layers in the fovea are maintained throughout life, whereas thickness of the inner retinal layers in other parts of the fundus may decrease in old dogs. Ofri & Ekesten (2019) have reported the thinning of NFL, outer and total retinal thickness in female beagles when puppies (<6 months) were compared to old dogs, but not when young females were compared to old dogs. However, their scans were ventral to the area centralis and the GCC thickness was not included as a parameter in their study.

Cheung *et al* (2017) reported GC-IPL macular thickness being more sensitive in identifying AD/MCI patients than NFL. Lim *et al* (2014), also discusses that the macular GCC could be more sensitive for MCI because synaptic density has a better correlation with cognitive impairment in AD, and the GCC thickness includes the inner plexiform layer, the dendrites of the ganglion cells and their synapsis with the bipolar cells. In our study, thinner temporal GCC

and nasal NFL were correlated with lower p-Aβ 42, whereas thinner foveal NFL was associated with higher p-Aβ 42. It is difficult to find a good explanation for why the NFL in adjacent areas would be related to effects of different polarity on p-Aβ 42 levels, but again the problems obtaining very precise measurements of the NFL thickness may have contributed, as well as the relatively small sample size. Furthermore, the correlation of parafoveal GCC measurements was considerably stronger than that between NFL and p-Aβ 42.

In our flash-VEPs , all frequently observed peaks and troughs in the recordings were assessed, even though previous reports on flash-VEPs in dogs only mention early wavelets up through the third positive component (P3). This was done with the purpose of exploring potential findings in a broader manner, especially since the focus of this study was changes in cortical function and later components of the VEP are considered to reflect extrastriate function better (Miller *et al*, 2005). Indeed, associations were seen in late components.

An age-related increase in time-to-peak was observed in most of the responses from P2. This agrees with the previous report by Kimotsuki *et al* (2006), of delayed time-to-peaks of P2, N2 and P3 seen with aging. They also reported decreased amplitudes for P2-N2 and N2-P3 with age, which was not observed in our study as an age effect was only seen on later peaks. The authors discussed the possibility of pathophysiological changes in the brain, such as cholinergic loss, as a probable explanation, as neurotransmitter homeostasis disturbances might be associated with delayed P2 in humans with AD but did not use other parameters to assess cognition.

In another study, Kimotsuki *et al* (2005) observed a delay of P2 and disappearance of N2 and P3 after lesioning the lateral geniculate body, suggesting that those potentials are generated at or caudal to that anatomic site on the visual pathway. In our study, wavelets going until nearly 500 ms after stimulus were observed in a somehow consistent matter, which has not been previously reported. The origin of those potentials is still unclear, however, those potentials after P3 could be proposed to be driven from association areas and different visual processing regions beyond the occipital cortex.

For the effect on Aβ and the possible use of flash-VEP as CCD biomarker, positive wavelets enhanced fitting the models more than negative ones. The highest correlation was found in a model with P2 and P4 time-to-peaks. However, different polarities of the parameter estimates were seen for the two components. While delayed P2 time-to-peak was associated with higher Aβ 42 concentration, the opposite was seen with P4. It seems however that the feasibility of using those parameters on a practical scenario would be limited due to their small effect, as a considerable increase in P4 or P2 time-to-peak would only lead to a small increase/decrease in Aβ.

Time and funds were limiting this project, which meant that we could not pursue finding solutions, such as the development of an optimal protocol for Aβ peptide quantification. Collection of additional samples, possible comparison of ELISA kits from different

manufacturers, or even production of own customized ELISA kits could also be done. Still, our p-Aβ 42 data may indicate trends between animals and age groups. Nevertheless, a greater sample size, especially for geriatric dogs may also add information, as well as other measures of cognitive status and post-mortem assessment.

6. Conclusions

In this pilot study, we found that OCT retinal imaging, as well as visual-pathway-related electrophysiological tests are potential tools for investigating age-related changes in the dog. It can also be concluded that the need for validated methods for Aβ peptide quantification in dogs, as well as a better understanding of their dynamics with age and interactions with actual behaviour limited our ability to detect which animals that might have cognitive deficits in our study.

Even though it is appealing to search for a sole diagnostic tool to identify cognitive impairment in aging dogs, a multimodal approach with the combination of biomarkers, detailed history and/or behavioural testing and lastly necropsy findings, seems to be unavoidable for a proper assessment of the usefulness of inner retinal morphology and post-retinal responses to visual stimuli. Studies involving behaviour are complex and dealing with geriatric animals can pose further obstacles, as frequently found co-morbidities can act as confounders.

Popular Scientific Article

UNRAVELLING ALZHEIMER'S DISEASE: POSSIBLE ANSWERS FROM CANINE EYES

Dogs have been used as spontaneous models for human dementia for many years as they might spontaneously present a canine counterpart of Alzheimer's disease, referred to as canine cognitive dysfunction syndrome (CCD). Alzheimer's and canine cognitive dysfunction share many common aspects including effects on behaviour of humans and dogs, and disease mechanisms, such as the deposition of a protein called amyloid beta in the brain.

It has been proposed that Alzheimer's patients might have thinner retinas and prolonged waves in electrophysiological tests as the retina is considered an accessible extension of the brain and vision problems are often reported with Alzheimer's disease. In this study we aimed to investigate possible associations of the structure of the retina, combined with the electrical activity of the brain when the eye is stimulated with flashes of light, and decay in cognition. As this has also been investigated in human patients.

This was done by doing two tests in six young and six old beagles: in one test, called optical coherence tomography (OCT), we measured the thickness of layers in the retina of each dog and in the second test called flash-VEP, we measured the electric activity of the brain in response to flashes of light using electrodes placed on the scalp. This was compared with the amyloid beta levels of each dog.

We could observe some differences in both tests in old beagles compared to young ones. Older dogs presented thinner retinal layers and some of the electrical responses from the brain were smaller and later. However, the amount of the amyloid beta protein in plasma from blood alone didn't provide a proper identification of decreased cognitive abilities and therefore a more comprehensive study including behavioural testing or other forms of assessing cognition could give more answers to whether OCTs and flash-VEPs are sensitive markers for canine dementia.

Acknowledgements

I would like to say my most heartfelt thank you to all involved in this project, it was a great learning experience. To my supervisor, Björn Ekesten, for his patience, guidance and willingness to teach me. To Ulrika Sjösten, for all the help with the laboratory analysis and so much more, to Anna Svensson as assistant supervisor for support and directions at the laboratory, to Desire Ferrari for managing the laboratory Beagles' sedation, and to Emma Hörnebro, for preparing the dogs, answering questions, and helping with the collection of samples. A thank you also to Lena Ström, my examiner, for all I got to learn and the wonderful feedback given.

References

1. Adams, B., Chan, A., Callahan, H., Siwak, C., Tapp, D., Ikeda-Douglas, C., Atkinson, P., Head, E., Cotman, C.W. and Milgram, N.W., 2000. Use of a delayed non-matching to position task to model age-dependent cognitive decline in the dog. *Behavioural brain research*, 108(1), pp.47-56.
2. Azkona, G., García-Belenguer, S., Chacón, G., Rosado, B., León, M. and Palacio, J., 2009. Prevalence and risk factors of behavioural changes associated with age-related cognitive impairment in geriatric dogs. *Journal of Small Animal Practice,* 50(2), pp.87-91.
3. Beltran, W.A., Cideciyan, A.V., Guziewicz, K.E., Iwabe, S., Swider, M., Scott, E.M., Savina, S.V., Ruthel, G., Stefano, F., Zhang, L. and Zorger, R., 2014. Canine retina has a primate fovea-like bouquet of cone photoreceptors which is affected by inherited macular degenerations. *PloS one, 9*(3). doi: 10.1371/journal.pone.0090390
4. Bemis, A.M., Pirie, C.G., Lo Pinto, A.J. and Maranda, L., 2017. Reproducibility and repeatability of optical coherence tomography imaging of the optic nerve head in normal beagle eyes. *Veterinary ophthalmology*, 20 (6), pp.480-487.
5. Benzal, A.S. and Rodríguez, A.G., 2016. Recent developments in Canine Cognitive Dysfunction Syndrome. *Pet Behaviour Science*, (1), pp.47-59.
6. Bichsel, P., Jr, J.E.O., Coulter, D.B. and Brown, J., 1988. Recording of visual-evoked potentials in dogs with scalp electrodes. *Journal of veterinary internal medicine*, *2*(3), pp.145-149.
7. Borghys, H., Van Broeck, B., Dhuyvetter, D., Jacobs, T., de Waepenaert, K., Erkens, T., Brooks, M., Thevarkunnel, S., Araujo, J.A., 2017. Young to Middle-Aged Dogs with High Amyloid-β Levels in Cerebrospinal Fluid are Impaired on Learning in Standard Cognition tests. *Journal of Alzheimers Disease*. 56, pp. 763–774.
8. Braga-Sá, M.B.P., Barros, P.S.M., Jorge, J.S., Dongo, P., Finkensieper, P., Bolzan, A.A., Watanabe, S.S., Safatle, A.M.V., Braga-Sá, M.B.P., Barros, P.S.M., Jorge, J.S., Dongo, P., Finkensieper, P., Bolzan, A.A., Watanabe, S.S., Safatle, A.M.V. (2018). Retina assessment by optical coherence tomography of diabetic dogs. Pesquisa Veterinária Brasileira, vol. 38, pp. 1966–1971.
9. Chen, G.F., Xu, T.H., Yan, Y., Zhou, Y.R., Jiang, Y., Melcher, K. and Xu, H.E., 2017. Amyloid beta: structure, biology and structure-based therapeutic development. *Acta Pharmacologica Sinica*, 38(9), pp.1205-1235.
10. Cheung, C.Y.L., Ong, Y.T., Hilal, S., Ikram, M.K., Low, S., Ong, Y.L., Venketasubramanian, N., Yap, P., Seow, D., Chen, C.L.H. and Wong, T.Y., 2015. Retinal ganglion cell analysis using high-definition optical coherence tomography in patients with mild cognitive impairment and Alzheimer's disease. *Journal of Alzheimer's Disease*. 45(1), pp.45-56.
11. Colle, M.A., Hauw, J.J., Crespeau, F., Uchihara, T., Akiyama, H., Checler, F., Pageat, P. and Duykaerts, C., 2000. Vascular and parenchymal Aβ deposition in the aging dog: correlation with behavior. *Neurobiology of aging*, 21(5), pp.695-704.

12. Cummings, B.J., Head, E., Afagh, A.J., Milgram, N.W. and Cotman, C.W., 1996. β-amyloid accumulation correlates with cognitive dysfunction in the aged canine. *Neurobiology of learning and memory,* 66(1), pp.11-23.
13. Davis, P.R., Giannini, G., Rudolph, K., Calloway, N., Royer, C.M., Beckett, T.L., Murphy, M.P., Bresch, F., Pagani, D., Platt, T. and Wang, X., 2017. Aβ vaccination in combination with behavioral enrichment in aged beagles: effects on cognition, Aβ, and microhemorrhages. *Neurobiology of aging*, 49, pp.86-99.
14. Den Haan, J., Verbraak, F.D., Visser, P.J. and Bouwman, F.H., 2017. Retinal thickness in Alzheimer's disease: a systematic review and meta-analysis. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, 6, pp.162-170.
15. Dewey, C.W. Chapter 7: Encephalopathies. In: Dewey, C.W. and Da Costa, R.C., 2015. Practical guide to canine and feline neurology. John Wiley & Sons. pp.147 -154.
16. Fletcher, T.F. Canine Brain Atlas Glossary. In: Dewey, C.W. and Da Costa, R.C., 2015. Practical guide to canine and feline neurology. John Wiley & Sons. pp.646.
17. González-Martínez, Á., Rosado, B., Pesini, P., Suárez, M.-L., Santamarina, G., García–Belenguer, S., Villegas, A., Monleón, I., Sarasa, M., 2011. Plasma β-amyloid peptides in canine aging and cognitive dysfunction as a model of Alzheimer's disease. *Experimental Gerontology*. 46, pp.590–596.
18. Graham, K.L., McCowan, C.I., Caruso, K., Billson, F.M., Whittaker, C.J. and White, A., 2019. Optical coherence tomography of the retina, nerve fiber layer, and optic nerve head in dogs with glaucoma. *Veterinary ophthalmology*, 23, pp. 97-112.
19. Hamnilrat, T., Lekcharoensuk, C., Choochalermporn, P. and Thayananuphat, A., 2015. Flash Visual Evoked Potentials in Normal Pomeranian Dogs and Those with Canine Cognitive Dysfunction. *The Thai Journal of Veterinary Medicine*, 45(3), pp.323-329.
20. Hansson, O., Seibyl, J., Stomrud, E., Zetterberg, H., Trojanowski, J.Q., Bittner, T., Lifke, V., Corradini, V., Eichenlaub, U., Batrla, R. and Buck, K., 2018. CSF biomarkers of Alzheimer's disease concord with amyloid-β PET and predict clinical progression: A study of fully automated immunoassays in BioFINDER and ADNI cohorts. *Alzheimer's & Dementia*, 14(11), pp.1470-1481.
21. Hart, N.J., Koronyo, Y., Black, K.L. and Koronyo-Hamaoui, M., 2016. Ocular indicators of Alzheimer's: exploring disease in the retina. *Acta neuropathologica*, 132(6), pp.767-787.
22. Head, E., 2013. A canine model of human aging and Alzheimer's disease. *Biochimica et Biophysica Acta – Molecular Basis of Disease, Animal models of disease*. 1832, pp.1384–1389.
23. Head, E., McCleary, R., Hahn, F.F., Milgram, N.W. and Cotman, C.W., 2000. Region-specific age at onset of β-amyloid in dogs. *Neurobiology of aging*, 21(1), pp.89-96.
24. Head, E., Pop, V., Sarsoza, F., Kayed, R., Beckett, T.L., Studzinski, C.M., Tomic, J.L., Glabe, C.G., Murphy, M.P., 2010. Amyloid β-Peptide and Oligomers in the Brain and CSF of Aged Canines. *Journal of Alzheimers Disease*. 20, pp. 637–646.
25. Helb, H.M., Issa, P.C., Fleckenstein, M., Schmitz-Valckenberg, S., Scholl, H.P.N., Meyer, C.H., Eter, N., Holz, F.G., 2010. Clinical evaluation of simultaneous confocal

scanning laser ophthalmoscopy imaging combined with high-resolution, spectral-domain optical coherence tomography. *Acta Ophthalmologica*. 88, pp. 842–849.

26. Hernandez-Merino, E., Kecova, H., Jacobson, S.J., Hamouche, K.N., Nzokwe, R.N. and Grozdanic, S.D., 2011. Spectral domain optical coherence tomography (SD-OCT) assessment of the healthy female canine retina and optic nerve. *Veterinary ophthalmology*, 14(6), pp.400-405.
27. Kergoat, H., Kergoat, M.-J., Justino, L., Chertkow, H., Robillard, A., Bergman, H., 2001. An evaluation of the retinal nerve fiber layer thickness by scanning laser polarimetry in individuals with dementia of the Alzheimer type. *Acta Ophthalmologica Scandinavica* 79, pp.187–191.
28. Kimotsuki, T., Yasuda, M., Tamahara, S., Matsuki, N. And Ono, K., 2005. Topographic analysis of flash visual evoked potentials in dogs. *Journal of veterinary medical science*, *67*(9), pp.869-875.
29. Kimotsuki, T., Yasuda, M., Tamahara, S., Tomihari, M., Matsuki, N. And Ono, K., 2006. Age-associated changes of flash visual evoked potentials in dogs. *Journal of veterinary medical science*, 68(1), pp.79-82.
30. Landsberg, G., 2005. Therapeutic agents for the treatment of cognitive dysfunction syndrome in senior dogs. *Progress in neuro-psychopharmacology and biological psychiatry*, 29(3), pp.471-479.
31. Landsberg, G.M., Nichol, J. and Araujo, J.A., 2012. Cognitive dysfunction syndrome: a disease of canine and feline brain aging. *Veterinary Clinics: Small Animal Practice*, 42(4), pp.749-768.
32. Liao, H., Zhu, Z. and Peng, Y., 2018. Potential utility of retinal imaging for Alzheimer's disease: A review. *Frontiers in aging neuroscience*, 10, p.188.
33. Lim, J.K., Li, Q.X., He, Z., Vingrys, A.J., Wong, V.H., Currier, N., Mullen, J., Bui, B.V. and Nguyen, C.T., 2016. The eye as a biomarker for Alzheimer's disease. Frontiers in neuroscience, 10, p. 536.
34. Madari, A., Farbakova, J., Katina, S., Smolek, T., Novak, P., Weissova, T., Novak, M. and Zilka, N., 2015. Assessment of severity and progression of canine cognitive dysfunction syndrome using the CAnine DEmentia Scale (CADES). *Applied Animal Behaviour Science*, 171, pp.138-145.
35. Mazzatenta, A., Carluccio, A., Robbe, D., Giulio, C.D., Cellerino, A., 2017. The companion dog as a unique translational model for aging. Seminars in Cell Developmental Biology: Science communication in the field of fundamental biomedical research, 70, pp.141–153.
36. McKhann, G.M., Knopman, D.S., Chertkow, H., Hyman, B.T., Jack Jr., C.R., Kawas, C.H., Klunk, W.E., Koroshetz, W.J., Manly, J.J., Mayeux, R., Mohs, R.C., Morris, J.C., Rossor, M.N., Scheltens, P., Carrillo, M.C., Thies, B., Weintraub, S., Phelps, C.H., 2011. The diagnosis of dementia due to Alzheimer's disease: Recommendations from the National Institute on Aging-Alzheimer's Association workgroups on diagnostic guidelines for Alzheimer's disease. *Alzheimer's & Dementia,* 7, pp. 263–269.

37. McLellan, G.J., Rasmussen, C.A., 2012. Optical coherence tomography for the evaluation of retinal and optic nerve morphology in animal subjects: practical considerations. *Veterinary Ophthalmology*, 15, pp.13–28.
38. Mihevc, S.P. and Majdič, G., 2019. Canine Cognitive Dysfunction and Alzheimer's Disease–Two Facets of the Same Disease? *Frontiers in neuroscience*, 13. doi.org/10.3389/fnins.2019.00604
39. Miller, N.R., Newman, N.J., Biousse, V., Kerrison, J.B., 2005. Walsh and Hoyt's clinical neuro-ophthalmology. Lippincott Williams & Wilkins. pp. 2515-2517.
40. Milgram, N.W., Head, E., Zicker, S.C., Ikeda-Douglas, C., Murphey, H., Muggenberg, B.A., Siwak, C.T., Tapp, P.D., Lowry, S.R. and Cotman, C.W., 2004. Long-term treatment with antioxidants and a program of behavioral enrichment reduces age-dependent impairment in discrimination and reversal learning in beagle dogs. *Experimental gerontology*, 39(5), pp.753-765.
41. Neilson, J.C., Hart, B.L., Cliff, K.D. and Ruehl, W.W., 2001. Prevalence of behavioral changes associated with age-related cognitive impairment in dogs. *Journal of the American Veterinary Medical Association*, 218(11), pp.1787-1791.
42. Nešić, S., Kukolj, V., Marinković, D., Vučićević, I., Jovanović, M., 2017. Histological and immunohistochemical characteristics of cerebral amyloid angiopathy in elderly dogs. *Veterinary Quaterly*, 37, pp. 1–7.
43. Noh, D., Choi, S., Choi, H., Lee, Y. and Lee, K., 2017. Evaluation of interthalamic adhesion size as an indicator of brain atrophy in dogs with and without cognitive dysfunction. *Veterinary radiology & ultrasound*, 58(5), pp.581-587.
44. Ofri, R. and Ekesten, B., 2019. Baseline retinal OCT measurements in normal female beagles: The effects of eccentricity, meridian, and age on retinal layer thickness. *Veterinary ophthalmology*. doi: 10.1371/journal.pone.0090390
45. Odom, J.V., Bach, M., Brigell, M., Holder, G.E., McCulloch, D.L. and Tormene, A.P., 2010. ISCEV standard for clinical visual evoked potentials (2016 update). *Documenta ophthalmologica*, *133*, pp.1-9.
46. Ozawa, M., Chambers, J.K., Uchida, K. and Nakayama, H., 2016. The relation between canine cognitive dysfunction and age-related brain lesions. *Journal of Veterinary Medical Science*, pp.15-0624.
47. Rofina, J.E., Van Ederen, A.M., Toussaint, M.J.M., SecrŔve, M., Van Der Spek, A., Van Der Meer, I., Van Eerdenburg, F.J.C.M. and Gruys, E., 2006. Cognitive disturbances in old dogs suffering from the canine counterpart of Alzheimer's disease. *Brain research*, 1069(1), pp.216-226.
48. Rosado, B., González-Martínez, A., Pesini, P., García-Belenguer, S., Palacio, J., Villegas, A., Suárez, M.L., Santamarina, G. and Sarasa, M., 2012. Effect of age and severity of cognitive dysfunction on spontaneous activity in pet dogs–part 1: locomotor and exploratory behaviour. *The Veterinary Journal*, 194(2), pp.189-195.
49. Rosado, B., González-Martínez, Á., Pesini, P., García-Belenguer, S., Palacio, J., Villegas, A., Santamarina, G. and Sarasa, M., 2012. Effect of age and severity of cognitive dysfunction on spontaneous activity in pet dogs–Part 2: Social responsiveness. *The Veterinary Journal*, 194(2), pp.196-201.

50. Rózga, M., Bittner, T., Batrla, R. and Karl, J., 2019. Preanalytical sample handling recommendations for Alzheimer's disease plasma biomarkers. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, 11, pp.291-300.
51. Salvin, H.E., McGreevy, P.D., Sachdev, P.S. and Valenzuela, M.J., 2010. Under diagnosis of canine cognitive dysfunction: a cross-sectional survey of older companion dogs. *The Veterinary Journal*, 184(3), pp.277-281.
52. Salvin, H.E., McGreevy, P.D., Sachdev, P.S. and Valenzuela, M.J., 2011. The canine cognitive dysfunction rating scale (CCDR): a data-driven and ecologically relevant assessment tool. *The Veterinary Journal*, 188(3), pp.331-336.
53. Sánchez, D., Castilla-Marti, M., Rodríguez-Gómez, O., Valero, S., Piferrer, A., Martínez, G., Martínez, J., Serra, J., Moreno-Grau, S., Hernández-Olasagarre, B. and De Rojas, I., 2018. Usefulness of peripapillary nerve fiber layer thickness assessed by optical coherence tomography as a biomarker for Alzheimer's disease. Scientific reports, 8(1), pp.1-10.
54. Schmidt, F., Boltze, J., Jäger, C., Hofmann, S., Willems, N., Seeger, J., Härtig, W. and Stolzing, A., 2015. Detection and quantification of β-amyloid, pyroglutamyl Aβ, and tau in aged canines. *Journal of neuropathology and experimental neurology*, 74(9), pp.912-923.
55. Schütt, T., Pedersen, J.T., Berendt, M., 2018. Chapter 15 - The Domestic Dog as a Model for Human Brain Aging and Alzheimer's Disease, in: Ram, J.L., Conn, P.M. (Eds.), Conn's Handbook of Models for Human Aging (Second Edition). Academic Press, pp. 177–194.
56. Sims, M.H., Laratta, L.J., Bubb, W.J. and Morgan, R.V., 1989. Waveform analysis and reproducibility of visual-evoked potentials in dogs. *American journal of veterinary research*, *50*(11), pp.1823-1828.
57. Smolek, T., Madari, A., Farbakova, J., Kandrac, O., Jadhav, S., Cente, M., Brezovakova, V., Novak, M. and Zilka, N., 2016. Tau hyperphosphorylation in synaptosomes and neuroinflammation are associated with canine cognitive impairment. Journal of Comparative Neurology, 524(4), pp.874-895.
58. Strain, G.M., Jackson, R.M. and Tedford, B.L., 1990. Visual evoked potentials in the clinically normal dog. *Journal of veterinary internal medicine*, *4*(4), pp.222-225.
59. Tao, R., Lu, Z., Ding, D., Fu, S., Hong, Z., Liang, X., Zheng, L., Xiao, Y. and Zhao, Q., 2019. Perifovea retinal thickness as an ophthalmic biomarker for mild cognitive impairment and early Alzheimer's disease. *Alzheimer's & Dementia: Diagnosis, Assessment & Disease Monitoring*, 11, pp.405-414.
60. Tapp, P.D., Siwak, C.T., Gao, F.Q., Chiou, J.Y., Black, S.E., Head, E., Muggenburg, B.A., Cotman, C.W., Milgram, N.W. and Su, M.Y., 2004. Frontal lobe volume, function, and β-amyloid pathology in a canine model of aging. *Journal of Neuroscience*, 24(38), pp.8205-8213.
61. Tartaglione, A., Spadavecchia, L., Maculotti, M. and Bandini, F., 2012. Resting state in Alzheimer's disease: a concurrent analysis of Flash-Visual Evoked Potentials and quantitative EEG. *BMC neurology*, 12(1), p.145.

62. Torres, D. and Tovar, M., 2016. Clinical guideline for assessing flash visual evoked potentials in laboratory dogs and normal data for beagle dogs. *Scandinavian Journal of Laboratory Animal Sciences*, *42*, pp.1-8.
63. Vite, C.H., Head, E., 2014. Aging in the Canine and Feline Brain. Vet. Clin. North Am. Small Anim. Pract., *Advances in Veterinary Neurology* 44, 1113–1129.
64. Wright, C.E., Harding, G.F.A. and Orwin, A., 1986. The flash and pattern VEP as a diagnostic indicator of dementia. Documenta Ophthalmologica, 62(1), pp.89-96.
65. Wyatt-McElvain, K.E., Arruda, J.E. and Rainey, V.R., 2018. Reliability of the Flash Visual Evoked Potential P2: Double-Stimulation Study. *Applied psychophysiology and biofeedback*, 43(2), pp.153-159.