Curtin School of Population Health

The Effects of S-Allyl-Cysteine and Cannabidiol on Cerebral and Retinal Neurovascular Integrity and Cognitive Function in Type 2 Diabetes

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DECLARATION

To the best of my knowledge and belief this thesis contains no material previously published by any other person except where due acknowledgment has been made.

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university.

The research presented and reported in this thesis was conducted in compliance with the National Health and Medical Research Council Australian code for the care and use of animals for scientific purposes 8th edition (2013). The proposed research study received animal ethics approval from the Curtin University Animal Ethics Committee,
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ABSTRACT

Type 2 diabetes (T2D) is increasingly recognised as a significant contributor to cognitive decline and dementia risk. Early cognitive impairments in T2D, particularly memory deficits, are often associated with hippocampal and cortical atrophy. Research implicates cerebrovascular dysfunction, chronic neuroinflammation and oxidative stress as potential pathological mechanisms driving cognitive impairment. Consistent with the latter, recent studies highlight a neurovascular link between retinal health and cognitive function in T2D, with comprised retinal capillary integrity and corresponding neurodegeneration both associated with memory deficits.

In T2D, the integrity of the neurovascular unit (NVU) is compromised, affecting both the blood-brain barrier (BBB) and the blood-retinal barrier (BRB). Chronic activation of glial cells, including microglia and astrocytes, contributes to NVU dysfunction. Significant hallmarks of glial activation are inflammation and oxidative stress, which can drive DNA damage culminating in neuronal cell loss. These insights underscore the critical need for therapeutic interventions that can protect neurovascular integrity and suppress glial-mediated neuroinflammation to preserve cognitive and retinal health in T2D.

Nutraceuticals with anti-inflammatory and antioxidant properties, such as S-allyl-cysteine (SAC) and cannabidiol (CBD), offer promising, safe and cost-effective therapeutic strategies. SAC has demonstrated potential in enhancing spatial memory, providing neurotrophic support and attenuating glial activation in murine models of Alzheimer disease. Similarly, CBD exhibits broad therapeutic effects, including reducing inflammation through modulation of the endocannabinoid system and various receptor targets, offering cognitive and vascular protection in models of neurodegeneration. However, both SAC and CBD have not been investigated for their long-term neuroprotective effects in T2D. The combined use of SAC and CBD presents a unique opportunity for clinical application, particularly given the heterogeneity of T2D pathophysiology and the potential for synergistic therapeutic effects. However, effectiveness of treatment requires early intervention and in both cognitive and retinal comorbidities of T2D, early preventative treatments are essential.

The interconnected pathophysiology of the brain and retina in T2D not only allows for comprehensive assessments of neuroprotective agents but also enables us to investigate the predictive capacity of retinal biomarkers for cognitive dysfunction. Optical coherence tomography (OCT) provides valuable insight into retinal thickness changes, particularly diabetic neurodegenerative thinning within the ganglion cell complex (GCC). Research suggests that retinal thinning is reflective of neurodegenerative changes in the cortex and hippocampus. Indeed, understanding the correlation between retinal neurodegeneration and cognitive deficits promote early detection and improve the efficacy interventions with SAC and CBD.

This thesis posits that T2D induces cognitive dysfunction through neurovascular, inflammatory and oxidative mechanisms. It hypothesises that SAC and CBD therapies will mitigate diabetic neuropathophysiology and preserve cognitive function, with combinatory treatment offering greater therapeutic effects. It also suggests that retinal changes in T2D mirror brain pathology and neurodegenerative GCC thinning can serve as a predictor of cognitive decline.

Chapter 2 focuses on cognitive dysfunction and its link to retinal thinning. Research was conducted using the well-established leptin-receptor deficient db/db mouse model at 14 and 28 weeks, reflecting early and moderate phenotypes of T2D, respectively. Mice were assessed for short- and long-term memory function using the novel object recognition and passive avoidance tests, respectively, followed by retinal thickness measurements with *in vivo* OCT imaging. Subsequent *ex vivo* immunofluorescence assessments included BBB breakdown (IgG extravasation), microglial activation (Iba1), astrocyte reactivity (GFAP) and DNA oxidation (8OHdG) in the brain, with concurrent glial activation (Iba1 and GFAP) in retina wholemounts.

While a diabetic phenotype with CNS pathophysiology was evident at both experimental timepoints, moderately diabetic db/db mice at 28 weeks showed comprehensive CNS pathologies with impairments in memory functions and retinal neurodegenerative GCC thinning. These changes were accompanied by BBB leakage, glial activation and oxidative damage in the brain, with corresponding glial-mediated neuroinflammation in the retina.

Notably, retinal GCC thickness correlated with cognitive decline and elevated glial-mediated inflammation in both neural tissues, while a follow-up correlation study revealed that plasma inflammatory markers showed mild and inconsistent associations with CNS complications.

These findings extend our understanding of the concurrent neurodegenerative mechanisms impacting cognitive and retinal health in T2D and underscore the potential utility of retinal imaging as a diagnostic tool for cognitive decline.

Chapter 3 evaluates SAC and CBD, individually and combined, against metformin in preventing cognitive dysfunction and retinal pathology. Building upon the outcomes of Chapter 2, a preventative dietary intervention study spanning 23 weeks was designed with experimental groups: non-diabetic db/+ controls, diabetic db/db controls, CBD, SAC, SAC+CBD, and metformin. Long-term dietary provision of SAC and CBD in db/db mice exhibited promising outcomes in preserving memory and retinal health, with potential synergistic effects in vascular protection realised. Compared to SAC and CBD, metformin demonstrated similar effects in protecting retinal health and neurovascular integrity but was less effective in preserving memory function, reducing neuroinflammation and mitigating oxidative stress. The variable neuroprotective outcomes of metformin, especially when compared with SAC and CBD, suggest that metformin may not offer extensive protection. Collectively, these findings support the therapeutic potential of SAC and CBD in promoting CNS health in T2D.

Collectively, this thesis provides compelling evidence supporting the potential of retinal assessments as robust predictors of cognitive decline and natural biomolecules, SAC and CBD, in neuroprotection in T2D. Further exploration of these findings in clinical settings and addressing the potential future directions identified from our research findings (Chapter 4) holds the potential to improve health outcomes for T2D individuals with cognitive and retinal comorbidities.

ACKNOWLEDGEMENT OF COUNTRY

We acknowledge that Curtin University works across hundreds of traditional lands and custodial groups in Australia, and with First Nations people around the globe. We wish to pay our deepest respects to their ancestors and members of their communities, past, present, and to their emerging leaders. Our passion and commitment to work with all Australians and peoples from across the world, including our First Nations peoples are at the core of the work we do, reflective of our institutions' values and commitment to our role as leaders in the Reconciliation space in Australia.

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PUBLICATIONS INCLUDED IN THIS THESIS

This thesis includes a peer-reviewed publication and a manuscript provisionally accepted, with one published study in the appendix. The contributions of M. Majimbi, along with endorsements from co-authors and copyright authorisations are included in the appendix.

Chapter 2:

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LIST OF ABBREVIATIONS

2-AG	2-Arachidonoylglycerol
8-OHdG	8-hydroxy-2'-deoxyguanosine
AGE	Advanced Glycation End Product
Akt	Protein Kinase B
AMPK	AMP-activated protein kinase
ATP	Adenosine Triphosphate
BBB	Blood Brain Barrier
BRB	Blood Retinal Barrier
CBD	Cannabidiol
CNS	Central Nervous System
Db/db	Homozygote Leptin Receptor-Deficient Mouse Model
DNA	Deoxyribonucleic Acid
DVP	Deep Vascular Plexus
GCC	Ganglion Cell Complex
GCL	Ganglion Cell Layer
GFAP	Glial Fibrillary Acidic Protein
GPCRs	G-Protein Coupled Receptors
H_2O_2	Hydrogen Peroxide
HFF	High Fat and Fructose
HOMA-IR	Homeostatic Model Assessment for Insulin Resistance
Iba1	Ionized Calcium-Binding Adapter Molecule 1
IgG	Immunoglobulin G
IPL	Inner Plexiform Layer
INL	Inner Nuclear Layer
MAPK	Mitogen-Activated Protein Kinase
MHC-II	Major Histocompatibility Complex II
MMP	Matrix Metallopeptidase
MRI	Magnetic Resonance Imaging
NADH	Nicotinamide Adenine Dinucleotide

NOR	Novel Object Recognition
NF-κB	Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells
NPDR	Non-Proliferative Diabetic Retinopathy
Nrf2	Nuclear Factor Erythroid 2-Related Factor 2
NVU	Neurovascular Unit
O_2^-	Superoxide
OCT	Optical Coherence Tomography
OH	Hydroxyl Radicals
PA	Passive Avoidance
PDR	Proliferative Diabetic Retinopathy
PI3K	Phosphatidylinositol 3-Kinase
PKC	Protein Kinase C
PPARγ	Peroxisome Proliferator-Activated Receptor Gamma
RGC	Retinal Ganglion Cell
ROS	Reactive Oxygen Species
SAC	S-allyl-cysteine
STZ	Streptozotocin
SVP	Superficial Vascular Plexus
T2D	Type 2 Diabetes
THC	Δ9-Tetrahydrocannabinol
TyG	Triglyceride-Glucose Index

CHAPTER 1. LITERATURE REVIEW

"The diabetic patient, on his own part, complains of loss of memory and of poor ability to concentrate the attention"

(Miles & Root, 1922, p. 1)

1.1 TYPE 2 DIABETES: PREVALENCE AND IMPLICATIONS FOR PUBLIC HEALTH

Diabetes mellitus encompasses a spectrum of metabolic disorders characterised broadly by hyperglycaemia, affecting an estimated 537 million adults aged 20-79 worldwide (Ogurtsova et al., 2022). This alarming statistic represents a threefold increase since 2000 and is projected to reach 783 million by 2045 (Sun et al., 2022). Among the various subtypes, Type 2 diabetes (T2D) stands out as metabolically heterogeneous, constituting 90% of diabetes cases. Its hallmark feature, progressive insulin resistance, arises from defective insulin secretion by pancreatic beta (β)-cells and diminished insulin sensitivity in tissues such as the liver, skeletal muscle, and adipose tissue (Goldstein, 2002). T2D is associated with modifiable environment triggers, population aging, polygenic susceptibility, and exposure to harmful stimuli (Murea et al., 2012). The obesity epidemic, fuelled by a high-caloric diet and sedentary lifestyle, emerges as the most significant risk factor for T2D (Ahmad et al., 2022).

Despite its growing prevalence, T2D is significantly underreported, with over 300 million individuals worldwide remaining undiagnosed despite meeting clinical criteria (Ogurtsova et al., 2022). An additional 300 million people are classified as 'prediabetic,' characterised by impaired glucose metabolism and heightened risk for diabetes-related complications. This underdiagnosis exacerbates the burden of complications, particularly in the central nervous system (CNS), which has drawn increasing research interest due to its profound impact on quality of life and healthcare resources.

Cognitive dysfunction is among the most concerning CNS complications associated with T2D (Biessels et al., 2020a). However, the mechanisms underlying these impairments are poorly understood, and the lack of reliable biomarkers hampers early detection and intervention. Beyond cognitive decline, T2D-related CNS complications are evident in the retina. While diabetic retinopathy is well-documented, early signs of neurodegenerative pathology often go unnoticed until symptoms worsen, leading to progressive visual impairment (Sinclair & Schwartz, 2019). Current treatments for diabetic retinopathy have varying efficacy, invasive delivery methods and unfavourable side effects (Li et al., 2023). These challenges have prompted researchers to investigate early-stage retinal changes and its association of brain health in T2D.

This literature review aims to summarise the pathological underpinnings of T2D-associated cognitive dysfunction and its association with retinal neurodegeneration, focusing on shared mechanisms such as neurovascular injury and oxidative stress. The review also explores the therapeutic potential of plant-derived supplements, S-allyl cysteine (SAC) and cannabidiol (CBD), and the first-line anti-diabetic treatment, metformin, in preventing cognitive dysfunction and retinal neurodegeneration in T2D.

1.2 LINK BETWEEN T2D AND CENTRAL NERVOUS SYSTEM ALTERATIONS

The systemic metabolic dysregulation characteristic of T2D—including hyperglycaemia, insulin resistance, dyslipidaemia, and disruptions in adipokine function—has profound implications for CNS health (Galicia-Garcia et al., 2020). Together, these abnormalities contribute to neural injury through mechanisms such as vascular dysfunction, glial activation, and oxidative stress.

The brain and neural retina are among the body's most metabolically active tissues, making them especially vulnerable to the metabolic disruptions in T2D. Chronic hyperglycaemia compromises the barrier properties of cerebral and retinal vasculature, allowing peripheral inflammatory mediators to infiltrate the CNS (Rübsam et al., 2018; van Sloten et al., 2020). This neurovascular dysfunction is further exacerbated by insulin resistance, which impairs neuronal survival, cognitive function, and vision (Li et al., 2022). Moreover, impaired insulin action contributes to oxidative stress through alterations of downstream pathways (phosphatidylinositol 3-kinase (PI3K)/Akt and the mitogen-activated protein kinase (MAPK)). Insulin signalling influences lipid metabolism,

partly through inhibition of fatty acid release from triglycerides (Li et al., 2023). T2D is linked to hypertriglyceridemia, which contributes to neurotoxicity through ligand-mediated activation of microglia and exacerbation of redox imbalance. Taken together, these mechanisms of injury promote CNS complications in T2D.

Markers such as the Homeostatic Model Assessment for Insulin Resistance (HOMA-IR) and the Triglyceride-Glucose (TyG) index have been valuable tools for understanding T2D pathology and its complications (Laws et al., 2017; Selvi et al., 2021). . Both indices correlate with increased risk for complications, including those affecting the CNS (Alzamil, 2020; Pan et al., 2021; Selvi et al., 2021). Research from our laboratory has shown that elevated HOMA-IR and TyG scores are associated with neurovascular injury and cognitive deficits (Majimbi et al., 2023; Mamo et al., 2019), highlighting their potential as markers for early detection of CNS involvement in T2D.

The phenotypes of T2D are further amplified by adipose tissue dysregulation (Buie et al., 2019), with leptin resistance playing a key role in exacerbating metabolic dysfunction and promoting chronic inflammation (Dilworth et al., 2021). Leptin's central role in regulating appetite and energy balance is impaired in T2D, where elevated serum leptin levels fail to elicit normal physiological responses, contributing to obesity, insulin resistance, hyperphagia, and CNS complications (Dilworth et al., 2021; Rhea et al., 2017). The leptin receptor deficient (db/db) mouse model utilised in this PhD research reflects these pathologies, making it a valuable tool for studying T2D-related CNS dysfunctions (Bogdanov et al., 2014; Sheskey et al., 2021).

By linking metabolic and inflammatory mechanisms to CNS alterations, this section highlights the multifactorial nature of T2D-associated neural injury and provides a rationale for investigating therapeutic strategies targeting these interconnected pathways.

1.3 CNS COMPLICATIONS ASSOCIATED WITH T2D: COGNITIVE DYSFUNCTION

The link between diabetes and progressive cognitive dysfunction was first described in the 1920s (Miles & Root, 1922). Subsequent research showed cognitive impairments in individuals with T2D, even after adjusting for factors such as age, smoking, hypertension, and heart disease (Biessels et al., 2020b; Ortiz et al., 2022). Notably, studies like the ACCORD-MIND trial revealed

that neurocognitive deficits in T2D primarily affect memory, psychomotor speed, attention and executive function (Ab-Hamid et al., 2023; Cukierman-Yaffe et al., 2009).

Early stages of cognitive decline in T2D can be subtle and challenging to detect, progressing from mild impairments in neuropsychological assessments to significant dysfunction impacting daily activities (Biessels & Despa, 2018). The progression of cognitive symptoms are associated with increased frequency of hypoglycaemic events, multi-organ comorbidities, and hospitalisation (Feil et al., 2011; Sinclair et al., 2022). Youth-onset T2D is particularly impactful, leading to worse cognitive scores compared to both nondiabetics and those diagnosed with T2D later in life (Brady et al., 2017). However, research on cognitive decline tends to focus on middle-aged and older adults with T2D (Li et al., 2016; Pelimanni & Jehkonen, 2019), neglecting younger populations.

T2D is recognised as an independent risk factor for developing dementia and neurodegenerative disorders, including Alzheimer's disease and Parkinson's disease (Verdile et al., 2015). The Rotterdam Study reported a relative risk of 1.9 (95% confidence interval (CI): 1.3–2.8) for vascular dementia in individuals with T2D (Ott et al., 1999). Recent meta-analyses, comprising more than 100 prospective studies and over 1 million research participants, confirmed that T2D increases the risk of incident dementia and Alzheimer's disease (Xue et al., 2019; Zhang et al., 2017). Interestingly, dementia risk was found to be elevated in groups with improved glycaemic status, including T2D patients treated with metformin or pre-diabetic cohorts.

While the onset and severity of cognitive dysfunction are correlated with T2D duration (Srikanth et al., 2020), notable impairment in hippocampal-dependent memory has been observed in individuals recently diagnosed with T2D (Van Gemert et al., 2018). Gold et al., (2007) found that T2D patients with good glycaemic control and less than 10 years of history showed deficits primarily in memory tasks, which coincided with a selective reduction in the volume of the hippocampal formation- a complex brain structure within the medial temporal lobe involved in memory formation and spatial navigation. These findings suggest that memory function may be particularly vulnerable in T2D and impairments within this domain are associated with neurodegeneration detected *via* neuroimaging techniques.

1.3.1 Neurological correlates of T2D associated cognitive dysfunction

Increasing evidence associates cognitive dysfunction with neuroanatomical and functional deficits in key brain regions in T2D (Moran et al., 2013; Sinclair et al., 2022; Zhang et al., 2016). Magnetic resonance imaging (MRI) studies have shown disrupted functional connectivity and neurodegeneration in the cortex and hippocampus (Gold et al., 2007; Lopez-Vilaret et al., 2022; H. Zhou et al., 2010). Additionally, cognitive symptoms in T2D have been linked to microinfarcts and white matter abnormalities indicative of neurovascular breakdown (Huang et al., 2021; Zhang et al., 2016). Post-mortem histological assessments demonstrated that clinical history of cognitive decline corresponded to elevated cerebrovascular disease and neurodegenerative lesions (Sheikh et al., 2022). However, the capacity to examine T2D-associated pathophysiologies are limited in humans. Hence, animal models have been critical to our understanding and continue to be used to explore the neurological correlates of cognitive dysfunction, particularly with the utility of specialised functional tests.

1.3.2 Murine studies of cognitive dysfunction and its central pathophysiology in T2D

Preclinical investigations into cognitive impairment associated with T2D consistently demonstrate deficits in hippocampal-mediated learning and memory functions (Carús-Cadavieco et al., 2023). For instance, diabetic db/db mice at 18 and 26 weeks exhibited reduced short-term episodic memory, as assessed by the novel object recognition (NOR) test, which interrogates innate exploratory behaviour (Zhang et al., 2021). Additionally, using the passive avoidance (PA) test, db/db mice at 16 weeks displayed impairments in long-term fear memory response (Ma et al., 2015). In the study by Yermakov et al., (2019), despite no discernible differences in working memory using the radial maze, db/db mice at 26 weeks showed cognitive inflexibility in the Morris water maze that corresponded with elevated *ex vivo* markers of neurodegeneration and axonal pathologies in the prefrontal cortex and hippocampus. These studies suggest that the db/db model is reliable for presenting cognitive symptoms associated with T2D.

In other models of diabetes, mice treated with low doses of streptozotocin (STZ) to mimic early T2D phenotypes also exhibit significant deficits in NOR and PA tests (Piątkowska-Chmiel et al., 2022). Similarly, the spontaneous Goto–Kakizaki rat model of T2D has shown decreased spatial

working memory and exploratory behaviour, associated with proliferation of neuroinflammatory microglia and neuronal atrophy in cortical and hippocampal regions (Allen et al., 2019; Girault et al., 2019; Hachana et al., 2020). Taken together, preclinical studies provide evidence that cognitive dysfunction in T2D coincides with cerebrovascular disruption, chronic glial activation, neuroinflammation and oxidative injury, which promote neurodegeneration. These findings support the association between cognitive dysfunction and CNS-specific neurodegenerative injury that is reported in clinical studies.

Unfortunately, assessing neurodegeneration in the brain usually requires costly MRI. However, degenerative disease within the CNS can also be evaluated in the retina. Indeed, the pathologies that contribute to neurodegeneration in the brain are mirrored in the retina in T2D, including compromised blood vessel integrity and reactive changes in glial cells. Studying retinal pathology may therefore serve as a surrogate marker for neurodegenerative changes in the brain, in addition to providing new insights into how T2D affects different parts of the CNS.

1.4 CNS COMPLICATIONS ASSOCIATED WITH T2D: RETINAL PATHOLOGY

The brain and retina share similarities in embryology and immune regulation and cellular metabolism, leading to similar pathological effects of diabetes on these tissues. Notably, retinal pathology can serve as an early indicator of cognitive symptoms and associated disease mechanisms (Garzone et al., 2023). Therefore, examining the retina is essential for understanding both ocular and cerebral disorders because "no other part of the central nervous system is amenable to direct observation" (Ptito et al., 2021, p. 7).

1.4.1 Overview of diabetic retinopathy

Diabetic retinopathy is the most common complication of T2D, with an estimated lifetime risk of 50-60% for patients (Wong et al., 2016). Non-proliferative diabetic retinopathy (NPDR) is characterised by vascular changes, including microvascular hyper-permeability and microaneurysm formation, leading to retinal ischemia and exacerbating inflammatory and neurodegenerative mechanisms (Cheung et al., 2010). Proliferative diabetic retinopathy (PDR) can develop with disease progression and is marked by neovascularisation as a response to ischemia.

These new vessels are fragile and can lead to vitreous haemorrhage, scarring and retinal detachment. However, the impact of T2D on the retina can be subtle, with early signs including neurodegeneration, persistent inflammation and subclinical microvascular disease (Wong et al., 2016).

1.4.2 Retinal neurodegeneration in T2D

Neurodegeneration represents an early stage of diabetic retinopathy and contributes to the clinical symptoms of NPDR or PDR (Sohn et al., 2016). This process primarily affects retinal ganglion cells (RGCs) and involves neuronal apoptosis due to persistent inflammation and neurovascular deterioration. The resulting loss of the RGCs leads to a reduction in the thickness of the inner neuroretina, where these neurons are located. As demonstrated in Figure 1.1, the ganglion cell complex (GCC) illustrates the topography of RGCs, with dendrites in the inner plexiform layer (IPL), cell bodies in the ganglion cell layer (GCL), and axons in the retinal nerve fibre layer (NFL) (Frydkjaer-Olsen et al., 2018). Unlike brain imaging techniques, optical coherence tomography (OCT) provides a cost-effective way to non-invasively quantify neurodegenerative changes in retinal thickness with high specificity and sensitivity in ophthalmology clinics (De Clerck et al., 2015).

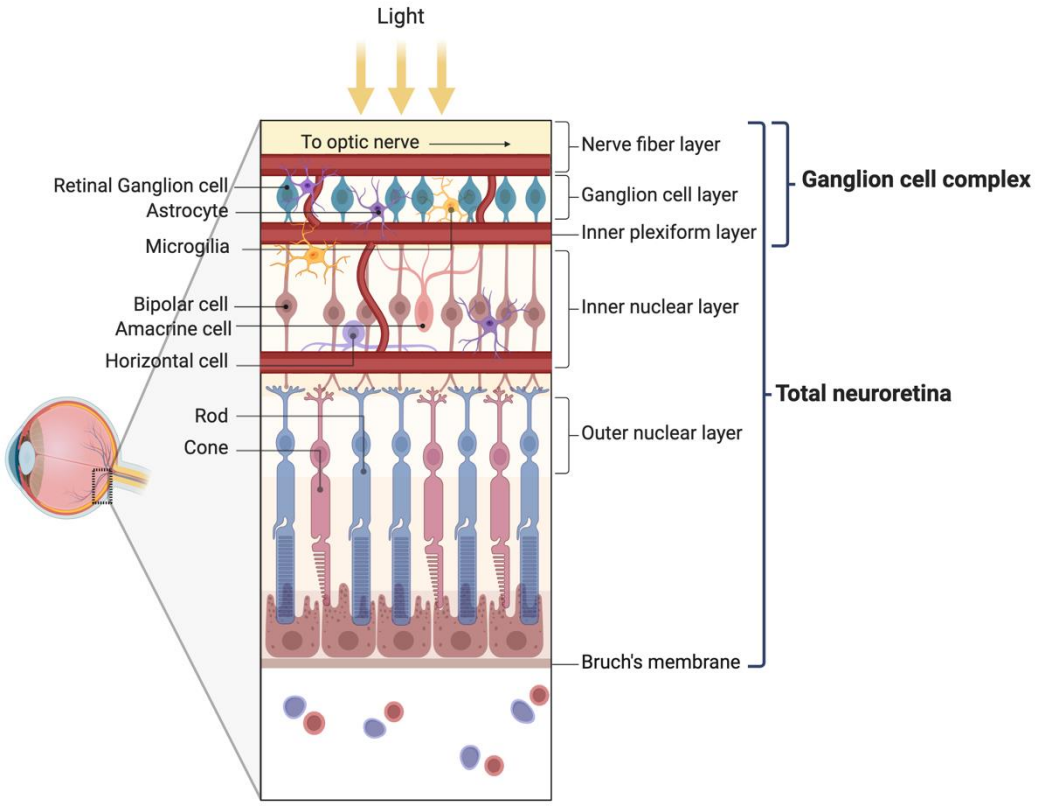

Figure 1-1 The retina topography with the layers of the GCC identified.
Made with Biorender.

A longitudinal OCT study by van Dijk et al., (2012) revealed a progressive reduction in IPL+GCL thickness in T2D patients with minimal retinopathy symptoms, beginning in the pericentral retina and extending peripherally. This study also noted a subtle reduction in the inner nuclear layer (INL) thickness, suggesting that T2D-related cellular integrity loss affects various retinal neuronal subtypes. Akin to observations in the brain, Yamada et al., (2021) demonstrated that diabetic reductions in GCC thickness coincide with features of polyneuropathy, indicating broader nerve cell dysfunction. Importantly, the thinning of the inner retina is significantly associated with diabetes duration, highlighting the progressive nature of neurodegeneration in T2D (Barber & Baccouche, 2017). Ferreira et al., (2016) supported these findings, reporting progressive thinning of the total neuroretina and GCC in diabetic patients, regardless of clinical 'vascular' retinopathy symptoms. However, the presence of retinal vasculopathy exacerbated neurodegeneration. These clinical findings suggest that T2D retinal thinning, which preferentially impacts the GCC,

manifests prior to overt symptoms of retinopathy. Therefore, investigating its underlying phenotype and potential therapeutics is of critical importance in preventing T2D CNS complications in the retina.

In animal studies, retinal neurodegeneration has been confirmed using *in vivo* OCT imaging. The mechanisms contributing to nerve cell loss have been corroborated with *ex vivo* immunofluorescence techniques. GCC thinning has been observed in diabetic db/db mice (Bogdanov et al., 2014; Majimbi et al., 2023) and STZ mice (Sergeys et al., 2019). In diabetogenic rats with spontaneous genetic mutations (for example, Zucker fatty and OLETF), neurodegeneration detected using *in vivo* OCT imaging was supported by *ex vivo* findings of decreased neuronal elements and an increased abundance of apoptotic caspase markers (Szabó et al., 2017; Yang et al., 2013).

1.4.3 Association between retinal pathology and cognitive dysfunction in T2D

The association between diabetic retinopathy and cognitive dysfunction has been established in longitudinal and cross-sectional studies of clinical T2D (Hugenschmidt et al., 2014). For example, thinning in the NFL and GCL have been linked to lower scores in standardised neuropsychological tests (Pedersen et al., 2023). Ophthalmic conditions are associated with elevated risk of incident dementia and Alzheimer's disease due to the 'brain-retina axis' (Lee et al., 2019; Schrijvers et al., 2012). Even after adjusting for age, metabolic status, smoking, and apolipoprotein E genotype, retinal imaging has been shown to identify an increased risk for cognitive pathologies (HR 1.50, p value = 0.03).

Research published from our lab, presented in Chapter 2, not only added to the literature on retinal-brain pathology, but it delved deeper into the shared pathophysiology of neurovascular breakdown and glial inflammation in diabetic db/db mice (Majimbi et al., 2023). Evidence increasingly shows that retinal imaging and the assessment of clinical neurodegeneration could serve as a valuable screening tool for cognitive dysfunction and cerebral neurodegeneration (Allen et al., 2019). Further investigations of the mechanisms underlying both retinal neurodegeneration and cognitive

dysfunction may aid the identification of therapeutic targets for T2D-associated CNS complications.

1.4.4 Overview central mechanisms linking T2D to CNS alterations

The observed retinal neurodegeneration in individuals with T2D, as revealed by OCT, intricately links to cognitive dysfunction through shared pathophysiological mechanisms. Compelling evidence from both clinical and preclinical studies underscores T2D's profound impact on neurovascular and neuroinflammatory processes. A critical facet of cognitive dysfunction in T2D involves the breakdown of neurovascular integrity, encompassing both the blood-brain barrier (BBB) and the blood-retinal barrier (BRB). This breakdown results in the extravasation of blood-derived molecules, inflammatory agents, and immune cells into neural tissue, thereby triggering neuroinflammatory responses (Sims-Robinson et al., 2016). Moreover, metabolic dysregulation and systemic inflammation induce the remote activation of glial cells, exacerbating neurovascular pathology (Li et al., 2023).

Chronic microglial activation and astrocyte reactivity have been observed in the neurodegenerative processes of both the retina and the brain in individuals with T2D (Coorey et al., 2012; Richa et al., 2017). These chronically activated glial cells release pro-inflammatory cytokines and reactive oxygen species (ROS), leading to oxidative stress (Luc et al., 2019). This pro-oxidative environment can cause irreversible cellular damage, including DNA alterations, contributing to persistent CNS complications. Understanding the intricate roles of these pathogenic events will be vital for developing innovative treatment interventions.

1.5 NEUROVASCULAR DYSFUNCTION AND BARRIER PERMEABILITY IN T2D

The neurovascular unit (NVU) is a complex and dynamic system comprising neurons, glial cells (astrocytes, and microglia), and vascular components (endothelial cells, pericytes, and the basement membrane) (Lochhead et al., 2020). It forms the structural and functional basis of the BBB and BRB in the brain and retina, respectively. The NVU is pivotal for maintaining the homeostasis required for proper CNS function (Abbott et al., 2006; Klaassen et al., 2013). This

section provides an overview of the NVU's role in CNS health and its involvement in T2D-related cognitive dysfunction and neurodegeneration.

The cellular components of the NVU collaborate to maintain homeostasis within the brain and retina. Endothelial cells form the inner lining of the blood vessels and play a pivotal role in governing the exchange of substances between the blood and neural tissue (Abbott et al., 2006). Pericytes, nestled within the basement membrane, provide structural support, and help regulate vascular tone. Astrocyte end-feet enwrap approximately 99% of capillary vessels, constituting a secondary layer of the basement membrane (Volterra & Meldolesi, 2005). Healthy astrocytes are crucial for BBB/BRB integrity, as they regulate blood flow, permeability, ion balance, energy metabolism, and immune protection. Microglia and neurons located in the perivascular regions exert modulatory effects through the release of signalling mediators (Bogush et al., 2017). The myelin-producing oligodendrocytes, and their percussor cells, interact with the NVU to ensure neuron viability.

1.5.1 The importance of blood-brain barrier (BBB) and blood-retinal barrier (BRB) in CNS health

The NVU is integral in regulating cerebral blood flow, supplying nutrients and oxygen to neural tissue, and eliminating metabolic waste products (Yan et al., 2020). The BBB and BRB control the passage of molecules, ions, and cells between the blood and neural tissue. These barrier properties are crucial for safeguarding the CNS from harmful substances while permitting essential nutrients and signalling molecules to enter the neural microenvironment. The intact BBB/BRB permits the passive exchange of gases, small molecules with fewer than eight hydrogen bonds, and lipids weighing less than 600 Da (Banks & Greig, 2019). A compromised BBB/BRB, indicated by leakage, allows the uncontrolled movement of large macromolecules and inflammatory mediators into the brain and retinal parenchyma, leading to degenerative pathologies. A neurovascular axis for CNS health and disease has been examined in recently published reviews (Li et al., 2022), and authors conclude that glial cells play a critical role in barrier maintenance.

1.5.2 The role of neurovascular injury in CNS alterations

Neurovascular injury can encompass endothelial dysfunction with loss of tight junction proteins, pericyte dropout, and basement membrane abnormalities. In the context of T2D, astrocyte reactivity and microglial activation have been demonstrated to contribute to neurovascular injury, leading to observed BBB and BRB hyper-permeability in subjects with cognitive and/or retinal diseases, as depicted in Figure 1.2 (discussed in greater detail in Section 1.5.3).

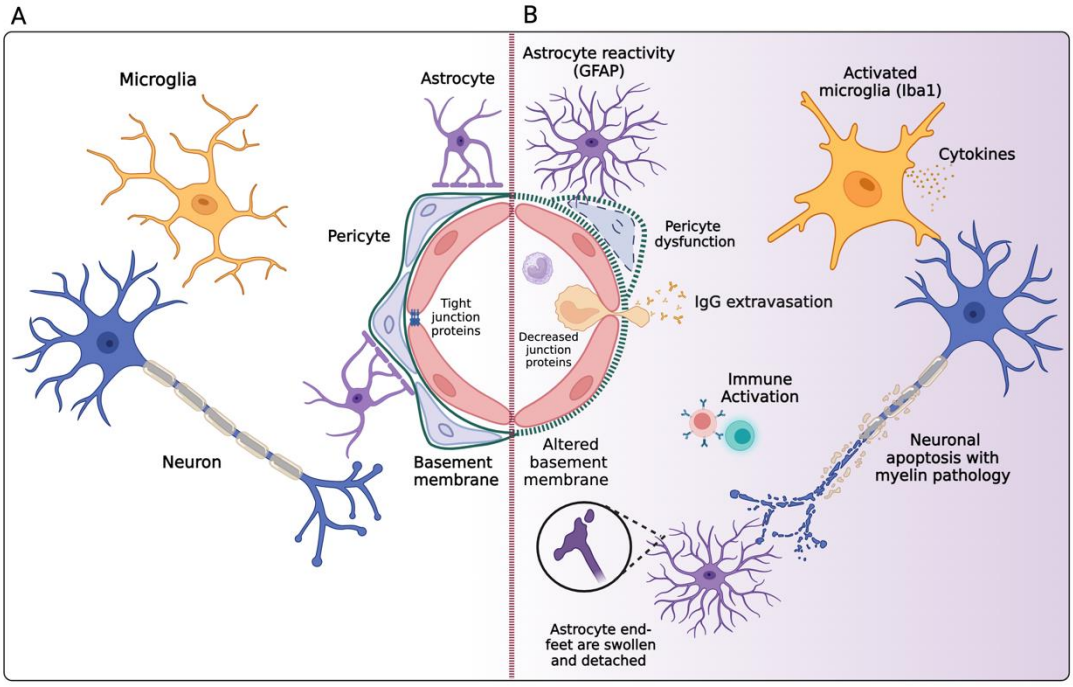

Figure 1-2 Schematic representation of the NVU in healthy CNS (A) and in the context of T2D (B).

Made with Biorender. (Adapted from Knox et al., 2022; Molecular Psychiatry)

Impairments in vascular-supporting functions of astrocytes and microglia can directly cause disruptions in the BBB and BRB. In T2D, dysregulated astrocytes impair BBB/BRB integrity through release of proteases such as matrix metallopeptidases (MMPs) that degrade basement membrane and endothelial junction proteins (Song et al., 2015). Additionally, astrocyte end-feet can become swollen and detached from microvessels, further damaging the NVU (Agrawal et al., 2006). Microglial activation in T2D is associated with increased proliferation around the perivascular space and indiscriminate phagocytosis of NVU cellular material, including astrocyte end-feet (Ramos-Rodriguez et al., 2015; Zhang et al., 2021). Chronic activation of microglia and

astrocytes in the brain is linked to ischemic-mediated neuronal loss and worsened cognitive outcomes (Li et al., 2023).

In the retina, reactive gliosis induces neurovascular lesions even before they are clinically detectable with retinal imaging techniques (Simó et al., 2018). The progression of retinal neurodegeneration is marked by inflammatory and ischemic-mediated neuronal loss. Within the retina, astrocytes residing in the GCC become reactive and induce lesions, particularly in the superficial vascular plexus (SVP) located in the RNFL (Bhatta et al., 2015). Additionally, the deep vascular plexus (DVP) located in the INL shows significant microglial-mediated structural derangements that promote leakage (Mills et al., 2021). Disruptions in both capillary networks, which form the inner BRB (iBRB), are associated with retinal neurodegeneration (Sinclair & Schwartz, 2019). Altogether, the deterioration of the NVU and loss of its 'selective barrier' properties, mediated by glial activation, leads to extravasation of blood-derived macromolecules, functional impairment, and degeneration.

1.5.3 Evidence of BBB/BRB leakage in T2D associated cognitive function and retinal neurodegeneration

Cerebrovascular leakage events have emerged as significant contributors to cognitive dysfunction and dementia in individuals with T2D (Groeneveld et al., 2018). Advanced imaging techniques using immunolabelled blood biomarkers, dyes and intravenously (IV)-administered contrast agents have provided crucial insights into vascular abnormalities in T2D (van de Haar et al., 2016; Yan et al., 2020). Notably, studies have reported evidence of BBB hyperpermeability, as indicated by a prolonged gadolinium tracer signal, even in well-managed T2D patients (Starr et al., 2003). Moreover, Shao et al., (2022) found a higher prevalence of cerebral microbleeds in T2D individuals with cognitive impairments compared to both non-cognitively impaired counterparts and nondiabetic controls. Additionally, post-mortem analyses using 960 Da Evans blue dye have revealed increased tissue penetrance in the brains of individuals with T2D, highlighting the clinical relevance of BBB dysfunction in this population (Sheikh et al., 2022).

Preclinical studies have further elucidated the role of BBB dysfunction in T2D-related cognitive decline. For instance, in adult rhesus monkeys with spontaneous T2D, *in vivo* Dynamic Contrast-Enhanced MRI (DCE-MRI) and *ex vivo* immunofluorescence analysis of extravascular 150 kDa immunoglobulin G (IgG) have confirmed BBB leakage, emphasising the translational relevance of these techniques (Xu et al., 2017). Notably, studies from our lab group have shown elevated IgG extravasation in C57BL/6J mice on a long-term high fat and fructose (HFF) diet (Takechi et al., 2017). These findings highlight the robustness of BBB hyperpermeability as an observable pathophysiological hallmark of cognitive dysfunction across different experimental paradigms.

While the neurovascular pathology in the retina has been more substantially investigated, studies have typically focused on the characterisation of end-stage retinopathic leakage. However, emerging evidence suggests that subtle BRB disruptions occur in the early stages of T2D CNS complications and are associated with glial activation and neurodegeneration (Madeira et al., 2021). Studies in animal models, such as a pig model of T2D, have shown that focal BRB leakage, astrogliosis, and loss of RGCs are associated with neurodegenerative thinning in the inner retina (Acharya et al., 2017). Chronically active microglia also contribute to BRB leakage, as they are the primary source of metabolic stress in retinal vascular disease (Bhatta et al., 2015).

In conclusion, BBB/BRB leakage can be considered as hallmarks of cognitive dysfunction and retinal neurodegeneration in T2D. The loss of neurovascular integrity is known to exacerbate neuroinflammation, which further promotes neurovascular breakdown in a viscous cycle. The next section will further explore the role of glial-mediated inflammation in T2D-related CNS alterations.

1.6 GLIAL ACTIVATION, INFLAMMATION AND OXIDATIVE STRESS IN T2D

1.6.1 Microglial activation in T2D

Microglia are resident immune cells in the CNS that constantly monitor the microenvironment and respond to various stimuli. Under physiological conditions, their activity is regulated by glycoproteins secreted by astrocytes, vascular endothelial cells and neurons, which down-regulate inflammatory responses and maintain tissue integrity (Shen et al., 2017). In the retina, retinal

ganglion cells (RGCs) are pivotal in regulating microglial activity through release of neurotrophic factors and interactions via cell-surface molecules (Fletcher et al., 2023), ensuring the preservation of retinal structure and function.

In the context of T2D, microglia become activated due to a range of factors including cellular redox imbalance, vascular insufficiency, astrocyte reactivity, neuronal excitotoxicity, and activation of membrane-bound receptors by toxic lipid metabolites and inflammatory mediators (e.g., glycation products, cytokines, chemokines, ATP, glutamate) (Fletcher et al., 2023). This chronic activation leads to an upregulation of classical pro-inflammatory markers such as ionized calcium-binding adapter molecule 1 (Iba1), major histocompatibility complex II (MHC-II) and cluster of differentiation (CD) proteins (Mei et al., 2019; Shi et al., 2018). Activated microglia also show elevated expression of the transcription factor nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) and the intracellular NLRP3 inflammasome, further exacerbating inflammation, neuronal loss, and vascular disruptions (De Biase et al., 2017; Xiao et al., 2021).

The pathological effects of microglial activation extend beyond the cellular level. Behavioural studies in T2D animal models have demonstrated links between heightened microglial activity and anxiety-like behaviours, as well as memory deficits observed in hippocampal-mediated tasks, such as the passive avoidance test (Tomassoni et al., 2020). In a high-fat diet (HFD)-induced insulin resistance model, microglial activation, marked by Iba1/MHC-II proliferation, was accompanied by reduced dendritic spine density and impaired hippocampal plasticity, correlating with cognitive decline (Hao et al., 2016).

Clinical and preclinical studies have demonstrated microglial activation in the retina of individuals with T2D (Xia et al., 2021; Zeng et al., 2008). Samples from diabetic retinopathy patients showed elevated Iba1-microglia expression, correlated with increased inflammatory markers in the vitreous humor (Hu et al., 2022). Similar findings were observed in diabetic murine models, where Iba1-microglial hypertrophy was correlated with reductions in inner retina thickness and elevated markers of apoptotic caspases (Arroba et al., 2016). Interestingly, microglial activation in the retina has been linked to cognitive impairment in non-obese individuals with T2D (Tanaka et al., 2022), suggesting a broader role beyond retinal pathology.

1.6.2 Astrocyte reactivity in T2D

Astrocytes are essential components of the CNS immune response, contributing significantly to its neuroprotective functions in response to acute inflammation and vascular damage through the secretion of neurotrophic factors (Li et al., 2022). Additionally, astrocytes fortify the NVU by creating a "neurochemical barrier", which restricts the movement of immune cells from blood vessels into neural tissue (Horng et al., 2017).

Astrocyte reactivity, indicated by higher levels of glial fibrillary acidic protein (GFAP), is an established pathophysiology of T2D and other neurodegenerative diseases (Yan et al., 2020). The impaired actions of reactive astrocytes correspond to pathologies in both the brain and retina, including NVU deterioration, cognitive impairments, and neurodegeneration (Fernández-Sánchez et al., 2015; Viegas & Neuhauss, 2021). For instance, reactive astrocytes can indirectly contribute to neuroinflammation by disrupting the glutamine-glutamate cycle, leading to imbalance of excitatory neurotransmission in T2D. This disruption has been linked to impaired synaptic plasticity in the hippocampus, resulting in deficits in spatial learning and memory (Amin et al., 2013). Indeed, studies have shown that suppressing astrocyte overactivity can reduce neuroinflammation and improve metabolic function in the CNS (Mamo et al., 2019; Rahman et al., 2020).

In summary, activation of both microglia and astrocytes has been associated with inflammatory damage and neurovascular complications in T2D. Immunofluorescent markers like Iba1 and GFAP have proven valuable for studying the activation of these glial cells in the context of neuroinflammation. Importantly, glial activation indicates oxidative stress in the CNS due to the highly oxidative phenotypes of reactive astrocytes and microglia. The next section will discuss the consequences of oxidative stress in the context of T2D CNS complications.

1.6.3 Oxidative Stress in T2D

In the diabetic environment, excessive glucose activates the polyol and hexosamine pathways and increases protein kinase C (PKC) isoforms (Galicia-Garcia et al., 2020; Yamagishi & Matsui, 2010). Hypertriglyceridemia, on the other hand, drives the beta-oxidation of free fatty acids, leading to lipotoxicity and metabolic stress. These processes overwhelm the mitochondrial respiratory chain, increasing the activity of electron donors such as nicotinamide adenine dinucleotide (NADH) (Xie et al., 2008). Surplus electrons interact with oxygen molecules to generate large amounts of superoxide (O_2^-), which is the primary ROS in T2D (Luc et al., 2019). Secondary reactions involving superoxide produce additional ROS, including hydroxyl radicals (OH) and hydrogen peroxide (H_2O_2). This oxidative upsurge is further reinforced by impaired insulin signalling, which signifies a reduced the capacity for detoxification. Furthermore, oxidative stress is driven by the formation of advanced glycation end products (AGEs) through non-enzymatic glucose interactions with proteins, lipids, and nucleic acids (DNA and RNA) (Turk, 2010).

Oxidative stress plays a central role in the degradation of NVU integrity, cognitive abilities, and retinal health in T2D (Mendonca et al., 2020; Sajja et al., 2017; Wang et al., 2020). While ROS are essential for normal neuroglial communication, their excessive production by glial and vascular endothelial cells overwhelms the antioxidant defence system (Wu et al., 2018). Research has shown that activated microglia contribute to oxidative stress by synthesising toxic AGE-albumin complexes. Prolonged activation of microglia and astrocytes also weakens the antioxidant capacity by suppressing the master antioxidant transcription factor, nuclear-factor erythroid 2-related factor 2 (Nrf2) signalling (Shen et al., 2017; Vargas-Soria et al., 2023). Glial activation has been linked to BBB/BRB leakage (Bogush et al., 2017; Mei et al., 2019), partly due to the downregulation of Nrf2-mediated antioxidant enzyme production (Zhao et al., 2019).

1.6.4 Evidence of oxidative DNA damage in T2D

Enhanced oxidative stress in T2D can been be assessed by the abundance of stable molecular by-products of oxidative DNA damage (Luc et al., 2019). The accumulation of 8-hydroxy-2'-deoxyguanosine (8-OHdG), formed by OH binding to guanine nucleosides, serves as a reliable

surrogate marker for the overall oxidative burden. These mutagenic DNA modifications propagate inflammatory and apoptotic mechanisms that contribute to CNS complications in T2D (Lodovici et al., 2015). Research has shown that 8-OHdG levels are correlated with hypertriglyceridemia and vascular pathology in individuals with impaired fasting glucose (Al-Aubaidy & Jelinek, 2013).

Within the CNS, elevated levels of 8-OHdG in the hippocampus correspond to BBB leakage and microglial activation in hypertensive rats (Takeuchi et al., 2015). Research from our lab corroborate these findings, showing that 8-OHdG is linked to hippocampal glial activation and memory impairments (Majimbi et al., 2023). Similarly, genetically-induced T2D mice exhibit a pathological elevation of DNA binding proteins, coinciding with oxidative injury and cerebral immune cell activation (Shi et al., 2018). Studies in both patients and animal models of T2D consistently demonstrate that early-stage retinal diseases and loss of RGCs are mediated by oxidative damage to mitochondrial DNA (Miller et al., 2020).

Taken together, the metabolic phenotype of T2D is closely associated with oxidative stress, which is considered both a precursor and an exacerbator of inflammation. However, there is comparatively less research on DNA oxidation in the CNS compared to other markers of redox imbalance. Furthermore, the understanding of DNA oxidative damage is still in the early stages. Future studies should consider investigating the impact of therapeutics on DNA oxidation and glial activation as key cellular drivers of oxidative stress in diabetic cognitive dysfunction. Utilising measures like 8-OHdG could provide valuable insights into the relationship between cognitive dysfunction, neurovascular deterioration, and neurodegeneration in T2D.

1.7 CONCLUDING REMARKS ON T2D PATHOPHYSIOLOGY

The pathophysiology of cognitive dysfunction in T2D is summarised in Figure 1.3. The pathophysiological processes associated with cognitive dysfunction in T2D are interconnected. Metabolic dysregulation, including hyperglycaemia, insulin resistance, elevated lipids, and obesity, induces widespread biochemical alterations that promote peripheral inflammation (IL-1β, IL-6, TNF-α). Within the CNS, neurovascular and glial pathologies, which contribute to oxidative stress and neuronal loss, emerge as crucial mechanisms in the development of cognitive and retinal complications in T2D. Thus, preserving BBB/BRB integrity, modulating glial cell activation, and

preventing oxidative injury are critical strategies for mitigating cognitive decline and retinal pathology in T2D.

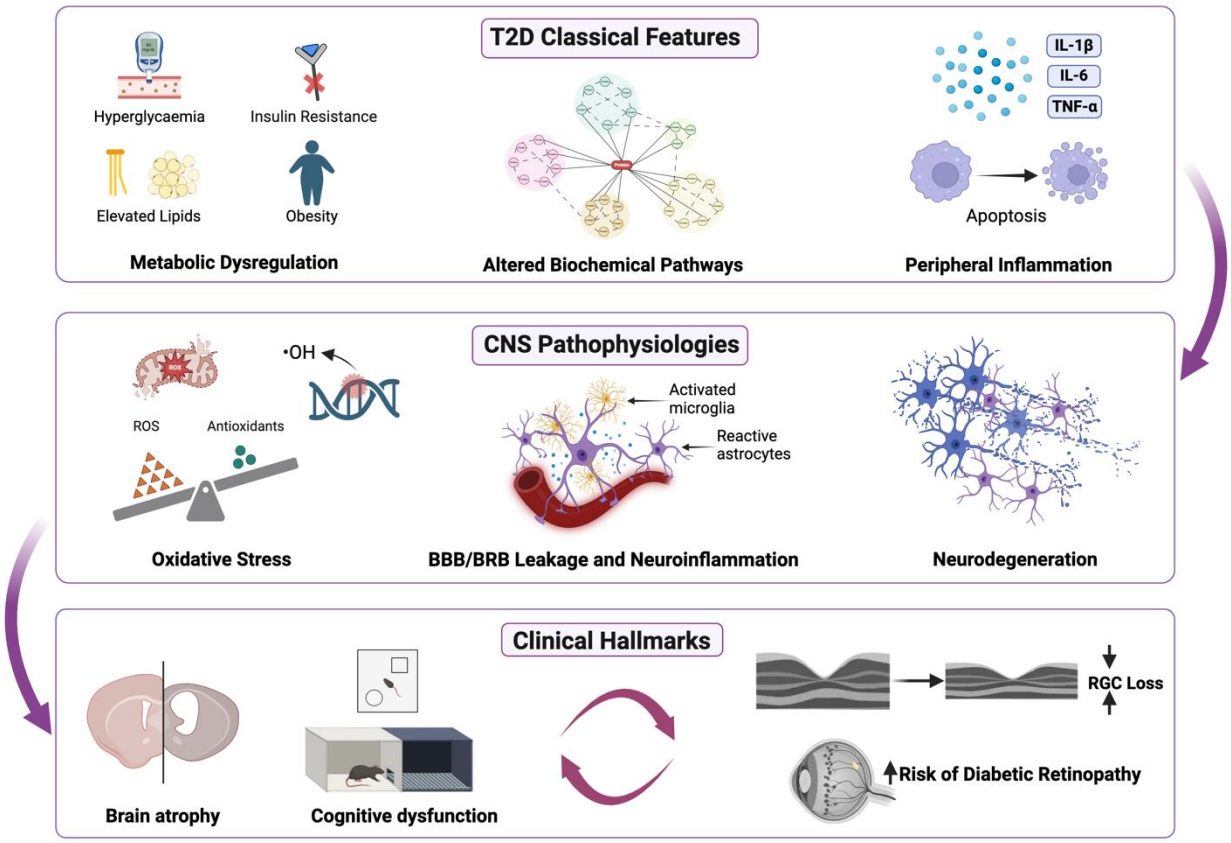

Figure 1-3 Summary of the interconnected pathophysiological processes associated with cognitive dysfunction in T2D.

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1.8 THERAPEUTIC POTENTIAL OF S-ALLYL CYSTEINE AND CANNABIDIOL

Naturally derived compounds with anti-inflammatory, antioxidant, and vasculoprotective properties have shown promise in various neurological and metabolic disorders (Brook et al., 2019; Madireddy & Madireddy, 2021). The following sections provide a comprehensive overview of the potential of natural therapeutics to improve T2D-associated CNS complications, with a specific focus on S-allyl-cysteine (SAC) and cannabidiol (CBD) as promising agents. Additionally, there is evidence suggesting a potential neuroprotective role for metformin, a conventional anti-diabetic

treatment. However, conflicting study outcomes warrant further investigations. Comparing conventional anti-diabetic treatments like metformin with natural molecules such as SAC and CBD present an intriguing avenue for research that could enhance patient outcomes.

1.8.1 Introduction to SAC

SAC is the most abundant organosulfur compound found in aged garlic extract. SAC accumulates through natural enzymatic processes facilitated by γ-glutamyl transpeptidase (γGTP) (Kosuge, 2020). SAC has a half-life of 10 hours and is excreted through the kidneys. Due to its small molecular size and high water solubility, SAC readily crosses the BBB and BRB (Ruiz-Sánchez et al., 2020). Research into the biological targets of SAC has unveiled its anti-inflammatory and antioxidant effects, along with its potential to improve energy metabolism (Colín-González et al., 2015). This diverse range of beneficial effects underpins its extensive utilisation in traditional Eastern healthcare practices and increasing popularity as a dietary supplement in Western culture.

1.8.2 Effects of SAC on the metabolic phenotype of T2D

SAC demonstrates an array of positive effects on metabolic dysregulation that is synonymous with T2D. Dietary provision of SAC at a 0.45% concentration was found to normalise various markers of metabolic syndrome in diabetic OLETF rats, including levels of plasma glucose, insulin, and triglycerides (Takemura et al., 2013). Additionally, SAC was reported to dramatically improve the lipid profile and serum free fatty acids in diet-induced obese mice (Zhai et al., 2018). These benefits suggest that SAC may prevent T2D complications by improving the diabetic metabolic status. A closer look at the literature shows the SAC also exerts protection within the CNS, through amelioration of glial pathology, restoration of redox balance, and preservation of neuronal viability.

1.8.3 Neuroprotective effects of SAC are relevant for T2D

The neuroprotective effects of SAC have been demonstrated in models relevant to T2D. In mice with LPS- inflammation and STZ- diabetes, SAC improved in recognition memory and amygdala-mediated fear response, accompanied by reductions in immunofluorescence markers of microglial

activation and astrocyte reactivity in the hippocampus (Baluchnejadmojarad et al., 2017; Zarezadeh et al., 2017). Additionally, chronic dietary administration of SAC (40 mg/kg) ameliorated cognitive deficits in senescence-accelerated SAMP8 and SAMP10 mice (Nishiyama et al., 2001). SAC also attenuated DNA oxidation in ischemic mice (Colín-González et al., 2011), suggesting its potential therapeutic advantage in various neurodegenerative pathologies.

In the retina, SAC protected against ischemic injury through neurovascular protective mechanisms, including the decrease in astrocyte-secreted proteinases known to erode the vascular membrane (Chen et al., 2012). A clinical study showed that SAC provision in the form of garlic supplementation improved retinal health in patients with T2D (Afarid et al., 2022). While it appears that SAC has positive effects in both the brain and retina in studies that resemble T2D CNS pathologies, direct evidence supporting this conclusion is limited.

1.8.4 Future research on SAC in T2D CNS complications

SAC has received minimal investigation in T2D-associated CNS complications, such as cognitive dysfunction or retinal neurodegeneration. Nonetheless, SAC shows promise as a treatment strategy for CNS disorders, either when administered alone or in combination with other bioactive molecules (Bokaeian et al., 2010; Takechi et al., 2013; Tsai et al., 2011). However, there is limited evidence, both in scope and the number of studies, supporting the effects of SAC when combined with other antioxidant and anti-inflammatory agents. Phytocannabinoids, known for exhibiting a broad range of therapeutic properties, may serve as interesting molecules to study alongside SAC. These molecules are chemically diverse, so understanding their individual and combined effects would be a novel area of research in diabetes.

1.8.5 Introduction to CBD

Cannabis sativa L. is a dioicous plant containing a diverse array of bioactive chemical entities, including over 150 phytocannabinoids as well as terpenes and flavonoids (Adams & Hunt, 1940). CBD is the primary non-intoxicating phytocannabinoid with a low oral bioavailability of 20% following extensive first-pass metabolism in the liver (Majimbi et al., 2021). Despite this, CBD

has garnered research popularity due to its potent anti-inflammatory and antioxidant properties (Peng et al., 2022).

The endocannabinoid system is shown to be dysregulated in neurodegenerative and inflammatory disorders, making CBD's modulation a significant therapeutic advantage (Atalay et al., 2019; Rapino et al., 2018). CBD is a negative allosteric modulator of cannabinoid (CB)1 and an inverse agonist of CB2 receptors (Charytoniuk et al., 2021). Unlike the psychoactive Δ9-tetrahydrocannabinol (THC), CBD does not induce the adverse effects and low tolerability associated with CB1 activation. CBD's low affinity modulation of CB1 and CB2 receptors is shown to downregulate immune cell migration and microglial proliferation, thereby mitigating proinflammatory responses (Ghasemi-Gojani et al., 2022; Sacerdote et al., 2005; Walter et al., 2003). Additionally, CBD influences the levels and immunomodulatory functions of endogenous cannabinoids, anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Peng et al., 2022; Saario & Laitinen, 2007).

1.8.6 Effects of CBD on the metabolic phenotype of T2D

CBD's pleiotropic effects stem from its complex pharmacology, which extends beyond the modulation of the endocannabinoid system. Among its many interactions with G-protein coupled receptors (GPCRs), CBD is an agonist of the transient receptor potential vanilloid-1 (TRPV1) and serotonin 5-hydroxytryptamine 1A (5-$HT1_A$) (Afshar et al., 2019), and an antagonist of the orphaned GPR55 (De Gregorio et al., 2019). These GPCRs are compromised in T2D, making them significant targets for therapy (Luo et al., 2019). Research suggests that modulation of GPR55, involved in cellular energy metabolism, contributes to CBD's therapeutic effects in immunometabolic disorders (Lipina et al., 2019). Importantly, CBD putatively binds to the peroxisome proliferator-activated receptor gamma (PPARγ), a nuclear transcription factor that's implicated in diabetes, obesity, and atherosclerosis (Esposito et al., 2011; Rangwala & Lazar, 2004; Villapol, 2018). CBD's agonism of PPARs, particularly the PPARγ isoform, holds great potential as an anti-inflammatory medicine in metabolic diseases (Villapol, 2018). Indeed, CBD has been shown to attenuate hypertriglyceridemia, and visceral adiposity (Bielawiec et al., 2020).

Moreover, CBD was reported to reduce peripheral inflammation and improved β-cell function (Gorelick et al., 2022; Weiss et al., 2008).

1.8.7 Neuroprotective effects of CBD are relevant for T2D

CBD's protective influence has been demonstrated in various models of CNS disease involving glial activation, BBB injury, and cognitive dysfunction. In a viral model of multiple sclerosis, CBD mitigated microglial activation and BBB leakage (Mecha et al., 2013). CBD treatment reduced astrocyte reactivity, protected against neuronal atrophy and improved learning abilities in murine models of Aβ-induced neuroinflammation (Esposito et al., 2011; Martín-Moreno et al., 2011). In rats with traumatic brain injury, both pre- and post-treatment with CBD improved neurological scores, prevented astrocyte hypertrophy and decreased BBB breakdown (Jiang et al., 2021). Furthermore, CBD attenuated microglial-mediated BRB leakage in retinas of animals with T2D and Alzheimer's disease phenotypes (Aires et al., 2019; Martín-Moreno et al., 2011; Sánchez-Fernández et al., 2023).

1.8.8 Future research on CBD in T2D CNS complications

Although CBD's therapeutic potential has been explored in various disorders, its application in T2D remains less studied. CBD has shown 'entourage effects' when administered with anti-inflammatory phytocannabinoids (Coles et al., 2022). However, CBD has not been readily studied alongside other medicinal plant biomolecules. Currently, it is unknown whether CBD, individually or as a combination treatment with organosulfur compounds, can ameliorate chronic inflammation, BBB disruption, cognitive dysfunction, and retinal changes in a model like the db/db mouse that is relevant to clinical T2D phenotypes.

1.8.9 Potential for SAC and CBD synergism and implications for T2D CNS complications

Combination therapies using plant and food-derived compounds have garnered interest for their potential synergistic effects in complex chronic diseases and cancer management (Businaro et al., 2021; Leena et al., 2020). Despite this trend, there has been limited investigation into the physiological effects of SAC and CBD outside their respective organosulfur and phytocannabinoid

families. However, studies have shown that when combined with other bioactive molecules, SAC and CBD can exhibit additive effects, suggesting a potential synergistic interplay. For example, co-treatment of SAC with the amino acid taurine amplified its lipid-modulating and renal function-preserving effects in diabetic animals (Rais et al., 2022). Similarly, combining CBD with a polyphenol compound demonstrated significantly enhanced anti-tumorigenic effects in an *in vitro* study (Yüksel et al., 2023).

Despite the lack of direct evidence, SAC and CBD possess molecular properties that make them suitable for co-treatment in CNS complications. Both compounds boast anti-inflammatory and antioxidant effects by inhibiting NF-κB and activating Nrf2 signalling pathways, respectively (Colín-González et al., 2015; da Silva et al., 2018; Vallée et al., 2017). Chronic glial cell activation, a hallmark of many CNS diseases, is mediated by the imbalance of the NFκB-Nrf2 transcription factors, and the combined effects of SAC and CBD may offer improved efficacy in regulating these pathways compared to monotherapy. Additionally, both SAC and CBD have demonstrated direct chelation of pro-oxidant species and the ability to reduce ROS-producing enzymes and pro-inflammatory cytokines (Hampson et al., 1998; Rodríguez Mesa et al., 2021).

In conclusion, SAC and CBD offer therapeutic promise for T2D CNS complications through their ability to target multiple molecular pathways. Their complementary mechanisms of action suggest the possibility of synergy, highlighting the potential for co-treatment in neurodegenerative diseases (Figure 1.4). The exploration of SAC and CBD, individually and combined, in T2D may be directly compared with to the effects of traditional anti-diabetic drugs. Future studies comparing the effects of SAC and CBD, individually and in combination, with traditional anti-diabetic drugs, such as metformin, could provide further insights into their therapeutic potential in T2D-related CNS complications.

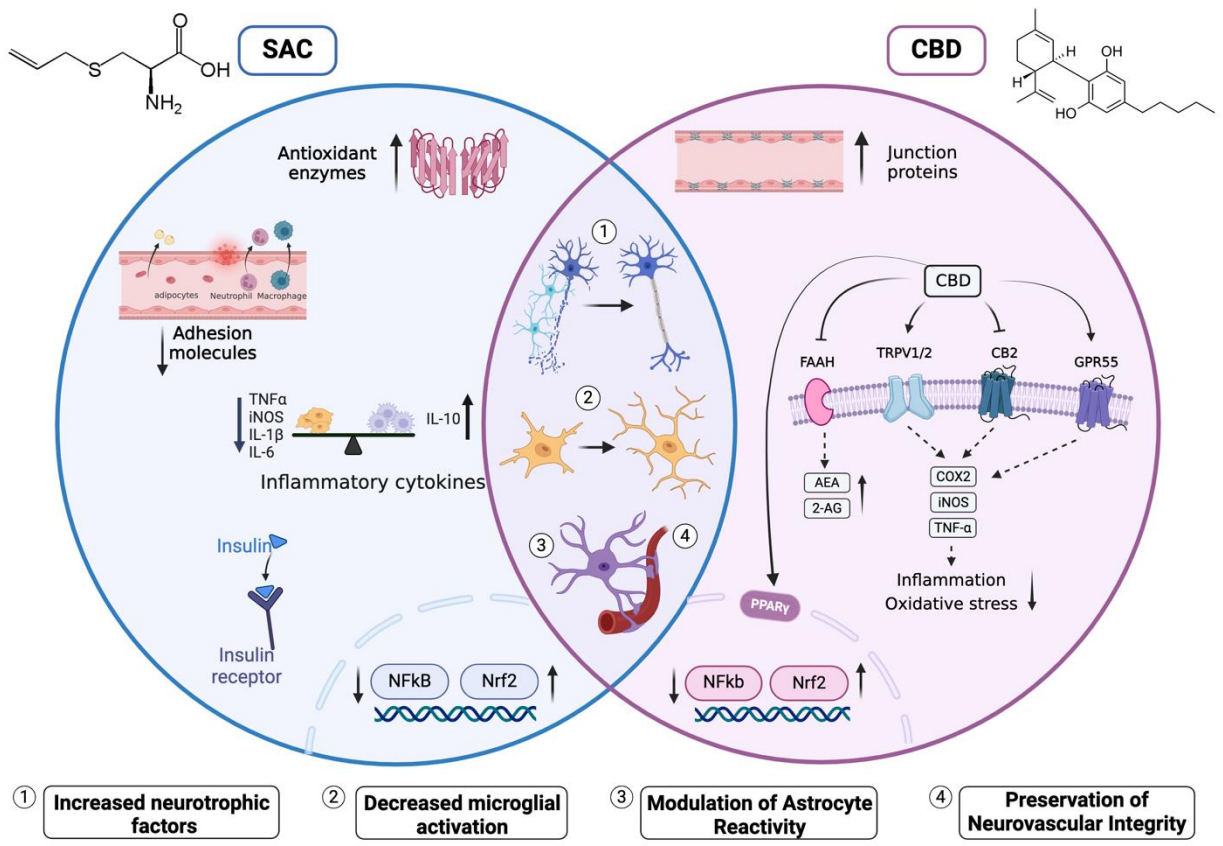

Figure 1-4 Schematic representation of the neuroprotective effects of S-allyl-cysteine (SAC) and Cannabidiol (CBD), and the potential for combinatory therapeutic effects.

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In summary, SAC is reported to have multiple effects, including reducing pathological adhesion to the endothelial lumen, increasing antioxidant enzyme capacity, and regulating inflammatory cytokine production. CBD, on the other hand, shows pleiotropic anti-inflammatory, antioxidative, and vasculoprotective effects primarily through its modulation of the endocannabinoid system and various receptor targets. Interestingly, the respective literature suggests that SAC and CBD may result in greater improvement of glial and vascular pathophysiologies upon combination treatment.

1.9 THERAPEUTIC POTENTIAL OF METFORMIN IN T2D CNS COMPLICATIONS

1.9.1 Introduction to metformin

Metformin is a first-line treatment of T2D derived from guanidine, a phytochemical isolated from the French lilac plant, Galega officinalis (Bailey & Day, 1989). The molecular properties of metformin involve blockade of respiratory chain complex 1, which reduces ATP production in the mitochondria. The subsequent decrease in energy status triggers activation of the energy sensor molecule, AMP-activated protein kinase (AMPK) (Pernicova & Korbonits, 2014). Through AMPK-mediated cascades, metformin reduces hepatic gluconeogenesis, promotes glucose uptake, and normalises PI3K/Akt signalling, a crucial pathway in insulin resistance. With its favourable safety profile and relatively low cost, metformin appears to be a pharmacological mainstay in managing metabolic dysregulation in T2D.

1.9.2 Neuroprotective effects metformin

Emerging evidence indicates that metformin exerts neuroprotective effects that are independent of glucose-lowering actions. Metformin treatment for 8 weeks correlated with improvements in learning, memory, and attention in a study of 20 non-diabetic individuals with either cognitive impairments or mild AD-induced dementia (Koenig et al., 2017). Similarly, an earlier cross-sectional study showed that metformin decreased mild cognitive impairment in the domains of memory, attention and information processing in individuals with diabetes (Y. Zhou et al., 2015). Studies from our lab have shown that in high-fat and high-fructose-fed mice, metformin prevented cognitive decline, astrogliosis and neurodegeneration (Mamo et al., 2019). Research using the APP/PS1 mouse model of Alzheimer's disease demonstrated that metformin's protective effects against chronic glial cell activation were associated with increased neuronal survival and preserved cognitive function (De Oliveira et al., 2016). Furthermore, metformin has demonstrated benefits in retinal health, preserving thickness and neurovascular integrity (Amin et al., 2022).

1.9.3 Mixed findings on the efficacy of metformin in T2D CNS complications

The neuroprotective effects of metformin have sparked debate, with recent large-cohort studies presenting conflicting evidence. While some studies support its therapeutic potential in addressing cognitive dysfunction (Zhang et al., 2022), others have failed to find conclusive evidence (Antal et al., 2022). Using pooled data from 5 population-based studies with 3,590 participants, the effects of metformin on cognitive outcomes and brain function appeared negligible, and even suggested potential for adverse impacts (Weinstein et al., 2019). Additionally, *in vitro* and *ex vivo* studies have reported that metformin may increase inflammation mediated by NF-κB and the production of ROS (Picone et al., 2015). These conflicting findings underscore the need for further investigation into the therapeutic potential of metformin in T2D CNS complications.

1.9.4 Future research on metformin in T2D CNS complications

There is accumulating evidence that metformin exhibits both glucose modulating and neuroprotective effects. However, investigations of metformin in T2D-assoctaed cognitive decline, neuroinflammation and retinal pathology are inconclusive. The db/db mouse model presents a unique opportunity to examine metformin's actions on metabolic and neurodegenerative phenotypes that characterise clinical T2D. Continued research efforts will be crucial in fully understanding the role of metformin in addressing the complex neurological complications associated with T2D.

1.10 CONCLUSION

The pathophysiological processes of T2D within the CNS encompass neurovascular breakdown, glial-mediated neuroinflammation, and oxidative stress. These processes culminate in neurodegenerative changes that underlie cognitive dysfunction and compromised retinal health. Notably, neurodegeneration in the retina is associated with cognitive deficits in individuals with T2D. Studies have demonstrated promising therapeutic potential for SAC and CBD in mitigating T2D-associated CNS complications. Both SAC and CBD exhibit additive effects when combined with other bioactive molecules, suggesting a potential synergistic interplay. However, the direct effects of combined SAC and CBD treatment remain unexplored. Future studies on T2D, utilising

clinically relevant models such as db/db mice, are crucial for further characterising CNS complications. Specifically, investigating neurovascular and glial pathologies and their contributions to cognitive dysfunction is essential. Additionally, future studies in db/db mice could establish associations between retinal pathophysiology and cognitive dysfunction through comprehensive clinical-based assessments of retinal structure alongside cognitive evaluations. Such studies would enhance our understanding of the interplay between retinal changes and cognitive deficits in the context of T2D. Lastly, exploring the therapeutic potential of SAC and CBD in T2D-associated CNS complications could yield valuable insights into targeted interventions for these complications.

THESIS AIMS

PhD Hypothesis

The central hypothesis of this research is that T2D induces cognitive dysfunction through pathological mechanisms that include neurovascular hyper-permeability, chronic glial activation, and oxidative stress. SAC and CBD therapies will mitigate diabetic neuropathophysiology and preserve cognitive function, with combinatory treatment offering greater therapeutic effects. Retinal changes in T2D mirror brain pathology and neurodegenerative GCC thinning can serve as a predictor of cognitive decline

Specific Aims

Aim 1: To investigate the pathophysiology of cognitive dysfunction in T2D using the LepR diabetic db/db mice and their nondiabetic db/+ counterparts at two critical timepoints: 14 weeks and 28 weeks of age.

Aim 2: To examine the association between retinal thickness and memory performance in the db/db model.

Aim 3: To investigate the therapeutic potential of naturally derived compounds like SAC and CBD, both individually and combined, in preserving CNS health in diabetic db/db mice. Moreover, to compare the efficacy of SAC and CBD against metformin in attenuating cognitive and retinal pathologies related to T2D.

The outcomes of this research are presented in Chapter 2 (Aims 1 and 2) and Chapter 3 (Aim 3). Chapter 2 also explores the association between diabetic plasma proinflammatory markers and cognitive dysfunction. Chapter 3 provides evidence that CBD, despite its known issues with low oral bioavailability, can be successfully administered through dietary intervention in db/db mice. A critical review of the results and considerations for future work are presented in Chapter 4. This

thesis includes one first-author publication as an appendix, which, although not directly corresponding to the main PhD objectives, presents significant preliminary work exploring CBD pharmacokinetics with microencapsulation techniques.

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CHAPTER 2. ASSESSING BIOMARKERS FOR COGNITIVE DYSFUNCTION IN T2D

2.1 SYNOPSIS

2.1.1 Background

T2D involves complex metabolic dysregulation and systemic inflammation, which can impact the CNS and lead to cognitive dysfunction (Biessels et al., 2020). Recent studies have highlighted retinal alterations as potential indicators of the neurodegenerative trajectory of cognitive disease in T2D (Sinclair et al., 2022). This chapter presents a study that investigates the CNS pathologies associated with T2D, focusing on memory impairments as early signs of cognitive dysfunction (Majimbi et al., 2023). The study aims to understand the disease mechanisms linked to cognitive decline, including BBB disruption, glial activation, and oxidative DNA damage, and their relationship with retinal health as measured by OCT imaging.

Additionally, this chapter includes an exploratory study that examines the systemic inflammatory profile of T2D and its correlation with cognitive function. These investigations emphasise the multifaceted nature of T2D's effects on the CNS and advocate for the use of biomarkers to improve diagnostic and preventive strategies for cognitive decline.

2.1.2 Methods in brief

The study employed the LepR db/db mouse model, mimicking early and moderate stages of T2D (Figure 2.1). *In vivo* assessments included OCT imaging for retinal layer measures and evaluation of short-term and long-term memory functions. *Ex vivo* measures included metabolic indices in plasma of glucose, triglycerides, and insulin levels, with insulin resistance evaluated using HOMA-IR and TyG index calculations Brain sections were assessed with immunofluorescence for markers of glial inflammation, oxidative DNA damage and BBB integrity, leveraging a novel automated quantitative analysis developed by our team (Nesbit et al., 2021). Retinal wholemounts were also imaged for glial activation, highlighting the shared neural pathophysiology in T2D.

In the follow-up study, plasma from the mice was utilised to quantify a panel of 13 cytokines and chemokines.

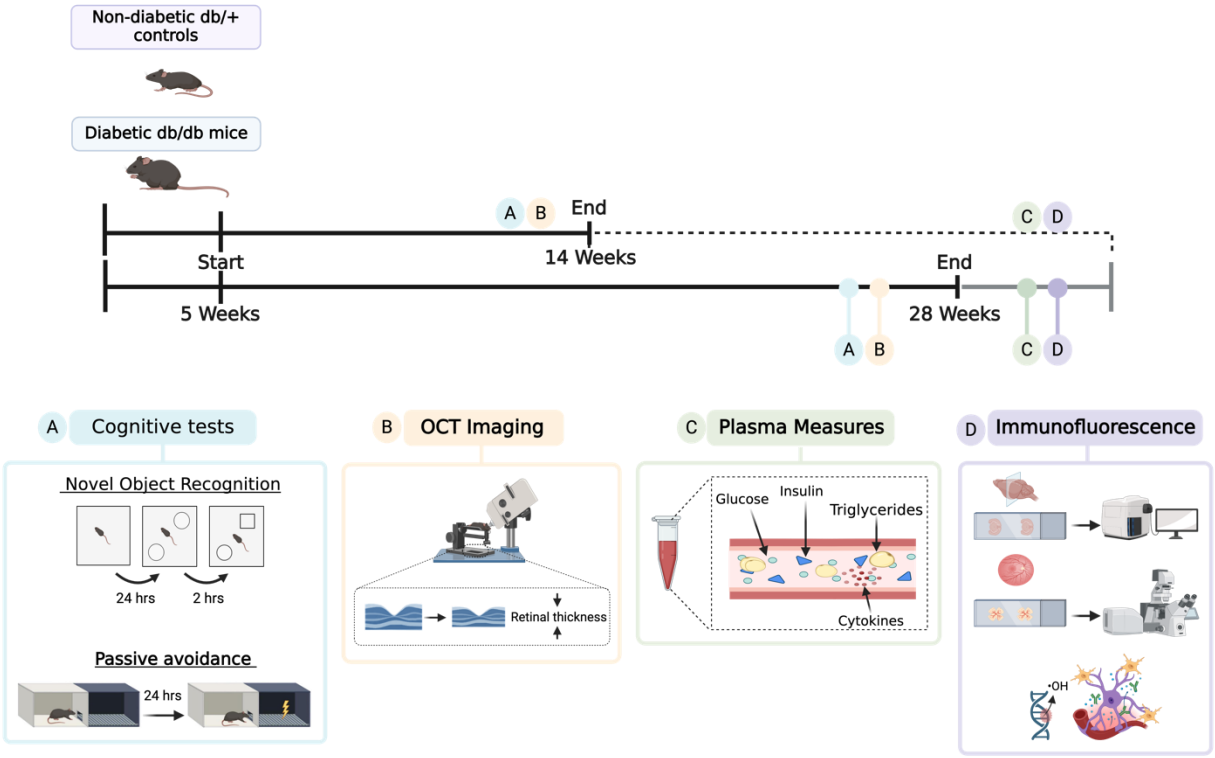

Figure 2-1 Experimental design for correlation studies.

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2.1.3 Results in Brief

Diabetic db/db mice showed marked inner retinal thinning that correlated with memory deficits, BBB leakage and glial-mediated neuroinflammation. Additionally, the smaller and exploratory study revealed a dynamic cytokine profile in T2D mice, reflecting both diabetic and age-related changes. The role of systemic inflammatory markers in predicting CNS complications remained inconclusive.

2.1.4 Discussion and Concluding Remarks

These findings support the potential of retinal imaging as a cost-effective marker for cognitive impairment in T2D. However, the impact of peripheral inflammatory markers on cognitive health requires further clarification. This research lays the foundation for future studies that could refine

a battery of CNS and peripheral biomarkers for early prediction of cognitive decline in T2D. Prospective research could include longitudinal studies with a broader range of cognitive assessments and comprehensive CNS pathophysiological measures. Overall, this work underscores the importance of the BBB and glial cells in developing neuroprotective strategies to preserve CNS health in individuals with T2D.

2.2 IN VIVO RETINAL IMAGING IS ASSOCIATED WITH COGNITIVE DECLINE, BLOOD-BRAIN BARRIER DISRUPTION AND NEUROINFLAMMATION IN TYPE 2 DIABETIC MICE

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In vivo retinal imaging is associated with cognitive decline, blood-brain barrier disruption and neuroinflammation in type 2 diabetic mice

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Introduction: Type 2 diabetes (T2D) is associated with chronic inflammation and neurovascular changes that lead to functional impairment and atrophy in neural-derived tissue. A reduction in retinal thickness is an early indicator of diabetic retinopathy (DR), with progressive loss of neuroglia corresponding to DR severity. The brain undergoes similar pathophysiological events as the retina, which contribute to T2D-related cognitive decline.

Methods: This study explored the relationship between retinal thinning and cognitive decline in the LepR *db/db* model of T2D. Diabetic db/db and non-diabetic db/+ mice aged 14 and 28 weeks underwent cognitive testing in short and long-term memory domains and *in vivo* retinal imaging using optical coherence tomography (OCT), followed by plasma metabolic measures and *ex vivo* quantification of neuroinflammation, oxidative stress and microvascular leakage.

Results: At 28 weeks, mice exhibited retinal thinning in the ganglion cell complex and inner nuclear layer, concomitant with diabetic insulin resistance, memory deficits, increased expression of inflammation markers and cerebrovascular leakage. Interestingly, alterations in retinal thickness at both experimental timepoints were correlated with cognitive decline and elevated immune response in the brain and retina.

Discussion: These results suggest that changes in retinal thickness quantified with *in vivo* OCT imaging may be an indicator of diabetic cognitive dysfunction and neuroinflammation.

KEYWORDS

diabetic cognitive decline, blood brain barrier, correlation, neuroinflammation, oxidative stress, retina neurodegeneration

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1 Introduction

Type 2 diabetes (T2D) induces significant cognitive decline and is an independent risk factor for Alzheimer's disease (1). Cognitive deficits in T2D encompass multiple domains, including spatial awareness, memory formation and executive function. These deficits can range from less-pronounced decrements to advanced impairment that resembles vascular and/or Alzheimer's dementia pathologies (2). The underlying and perhaps heterogenic mechanisms are largely unknown; however, evidence in preclinical dietary-induced T2D models (3) and in genetic LepR db/db mice (4) suggests that cerebral microvascular disruptions, chronic neuroinflammation and brain atrophy appear as crucial pathogenic events in T2D-related cognitive deficits. Cerebral microvascular disruption describes compromised blood-brain barrier (BBB) function, which is associated with the activation of microglia in brain tissue and infiltration of circulating leukocytes (5, 6). In animal models of T2D, these neurovascular changes precede memory impairment assessed by spatial learning challenges (3, 4, 7).

Determining cerebral microvascular disruption and neuroinflammation early may support a better cognitive trajectory in individuals living with T2D. Hyperpermeability of the BBB and neuroinflammation have been occasionally reported in patients with T2D using advanced magnetic resonance imaging (MRI)-based techniques (8).

The retina is neural-derived, and several lines of evidence suggest it may serve as a surrogate marker of cerebrovascular integrity (9). In T2D, significant changes in retinal thickness measured non-invasively using optical coherence tomography (OCT) are reported (10). Moreover, cross-sectional and longitudinal studies have shown that retinal thinning in T2D is associated with impairment in global cognitive function (11, 12). However, these studies did not consider a putative association between retinal thickness and cerebrovascular integrity per se. Thus, the objective of this study was to investigate whether changes in retinal thickness measured by OCT are associated with disruption to the BBB, neuroinflammation and cognitive decline in a well-established clinically relevant mouse model of T2D.

2 Materials and methods

2.1 Animals

Male mice with spontaneous homozygous mutation in the leptin receptor gene (db/db) and heterozygote *db*/+ of C57BLK/6J background were obtained from the Jackson Laboratory, the US and were maintained at the Animal Resource Centre, Western Australia. Animals at 4 weeks of age were group housed in a temperature-controlled laboratory at Curtin University on a 12 h light/dark cycle with standard chow (AIN93M, Specialty Feeds, WA) and water provided ad libitum. Following 7 d of acclimatisation, animals were randomly separated into 14-week and 28-week experimental endpoints. Body weight was measured prior to euthanasia. Experiments were conducted according to approved animal ethics protocol (Curtin Animal Ethics Committee, approval no. ARE 2018-19).

2.2 Cognitive assessment

2.2.1 Novel object recognition

The protocol for novel object recognition was adapted from a previous study (13). The set-up consisted of an open field box (45 cm x 45 cm x 40 cm) situated in a dimly lit room with a video camera that enabled movement tracking and automated data collection using HVS Image 2014 software (HVS Image, UK). During the first day, mice were habituated to the arena for 10 min without any objects. On the second day, two identical objects were placed in the arena in opposite corners approximately 10 cm away from the arena wall. Mice were exposed to the objects for 10 min during familiarisation phase, then placed back in their home cage. Animals that spent less than 10 s with each object were excluded from the study. An object was selected at random and replaced with a different, novel object. After 2 h, mice were returned to the arena for 5 min during the test phase. Exploration in the familiarisation and test phases was defined as sniffing the object. The arena and objects were regularly sprayed with 70% (v/v) ethanol to minimise odor cues. The preference index (PI) was calculated as the time spent exploring the novel object in the test phase relative to the combined time spent exploring both objects, summarised as: Preference index (PI)= Novel object[s]/(Novel object[s] + familiar object[s] x 100.

2.2.2 Passive avoidance

With a minimum of 24 h rest after the novel object recognition test, Passive Avoidance test was run according to a previous study (14) with several amendments, using step-through apparatus (Ugo Basile, Italy). Briefly, mice were placed in the illuminated chamber of the apparatus with the following experimental conditions for training phase: 1000 lux light intensity, 30 s door delay, 300 s step-through time, 2 s shock at 0.3 mA, and 10 s for the mouse to remain in the dark chamber prior to transfer to home cage. Animals that failed to enter the dark chamber during the training phase were excluded from the assessment. After 24 h, mice were returned to the illuminated chamber for the testing phase with identical experimental conditions, except the animals entered the dark chamber without a shock. Latency was calculated as the time taken to enter the dark chamber during the testing phase minus the time taken on training phase.

2.3 Optical coherence tomography

Retinal imaging was carried out according to a published protocol with minor alterations (15). Mice were anaesthetised using ketamine/xylazine (90 mg/kg ketamine, 12.5 mg/kg xylazine), and administered with eye drops containing 1% tropicamide and lignocaine (Akorn, Inc.) to dilate the pupils. Retinal imaging was conducted using an OCT imaging system

(Heidelberg Engineering, Germany) set to a 30° field of view. Mice were placed into the holding compartment and fitted with a custom contact lens to prevent formation of temporary cataracts. Volume scans were obtained of the posterior pole of the eye from the optic nerve head (ONH) to the retinal periphery. B-scan cross-sectional images were stored and thickness of the: 1) total neuroretina, 2) ganglion cell complex (including the nerve fibre, retinal ganglion cell and inner plexiform layers), and 3) inner and outer nuclear layers were measured around the ONH (central) and 400 µm superior to the ONH (mid-peripheral) in nasal-temporal regions. Mean thicknesses of each layer were averaged from 6 measurement points across each B-scan using FIJI software (ImageJ, US).

2.4 Euthanasia and sample collection

Following OCT imaging, deeply anaesthetised mice were exsanguinated *via* cardiac puncture and euthanised by cervical dislocation. The brain and right eye were removed from the cranium and prepared as follows: the left hemisphere of the brain was snap-frozen in liquid nitrogen immediately after dissection and stored at -80°C, whereas the right hemisphere of the brain and right eye were fixed in 4% paraformaldehyde overnight and cryopreserved in 20% sucrose for 3 d. The right eye was transferred to 0.1% sodium azide and stored at 4°C and the right hemisphere was frozen in isopentane/dry ice slurry prior to storage at -80°C. To prepare 20 µm coronal sections that contain both cortex and hippocampus regions, brains were embedded in Optimal Cutting Temperature medium and cut on the Leica CM1520 Cryostat onto Poly-Lysine coated slides (Tranjan).

2.5 Plasma measures for glucose, triglycerides, and insulin resistance

Blood obtained in EDTA-coated syringes was centrifuged at 4°C for 10 min at 4000 rpm. The supernatant plasma was aliquoted and stored at −80°C for further analysis. Non-fasting plasma glucose was measured at an optimised dilution of 1:100 using a commercial colorimetric assay kit (Abcam). Insulin levels were assessed with the Ultrasensitive Mouse Insulin ELISA kit (Mercodia). Finally, plasma triglycerides were measured using a colorimetric assay (Randox). All plasma measures were analysed according to the manufacturer's instructions. Insulin resistance was calculated using Homeostasis model assessment–insulin resistance (HOMA-IR) with the HOMA Calculator version 2.2.3 (Diabetes Trials Unit), and Triglyceride-glucose (TyG) index according to formula indicated below:

$$ln\left[triglycerides\,(mg/dL)\ \times\ glucose\,(mg/dL)/2\right]$$

2.6 Brain immunofluorescence

BBB integrity was investigated using an established protocol for quantifying extravasated plasma macromolecule, Immunoglobulin G (IgG) in the brain parenchyma (16). As reported in the protocol, sections were co-labelled with the capillary basement membrane marker, laminin α4 to improve identification of the microvascular boundary. Briefly, 20 µm coronal sections of snap frozen left hemisphere were fixed with 4% paraformaldehyde for 10 min at 20°C and non-specific binding sites were blocked in 10% donkey serum (ThermoFisher) for 30 min at 20°C. The sections were washed in 0.1 M phosphate buffer saline (PBS) and incubated with goat anti-laminin α4 (1:200, RnD Systems) in an antibody signal enhancer solution (17) overnight at 4°C. Sections were washed in PBS and incubated with donkey anti-goat IgG AlexaFluor 555 followed by goat anti-mouse IgG AlexaFluor 647 mixed in PBS, with each secondary incubation lasting 2 h at 20°C.

Brain neuroinflammation, glial reactivity and oxidative stress were assessed as described (14). Briefly, 20 µm thick fixed cryosections of the right hemisphere were rehydrated in PBS, and non-specific binding sites were blocked in 10% donkey serum (ThermoFisher) for 30 min at 20°C. Sections were incubated overnight at 4°C with either rabbit anti-ionized calcium-binding protein 1 (1:200, Iba1; Novachem) for microglia or a combination of goat anti-glial fibrillary acidic protein (1:500, GFAP; Abcam) for astrocytes and mouse anti-8-Hydroxyguanosine (1:500, 8-OHdG (15A3); Abcam) for DNA oxidation. Thereafter, sections were incubated with either goat anti-rabbit IgG AlexaFluor 488 (1:500, ThermoFisher) or a combination of donkey anti-goat IgG AlexaFluor 555 (1:1000, ThermoFisher) and 1:500 donkey anti-Mouse IgG AlexaFluor 647 (ThermoFisher) for 2 h at 20°C. DAPI was used to counterstain the nuclei.

Immunostaining of the cortex and hippocampus were captured at 20x magnification on the Zeiss Axioscan Z1 slide scanner (Carl Zeiss, Germany). Images were processed offline using Zeiss Zen Desk software, with Intellesis trainable segmentation module for leakage analysis (16). Semi-quantitative analysis of protein expression was determined using voxel intensity per volume for each region of interest.

2.7 Retina immunofluorescence

Retina immunofluorescence staining and imaging were carried out according to an optimised protocol developed in-house. To summarise, retinas were enucleated from mouse eyes in a petri dish containing 1XPB then transferred to a 96 well-plate, and incubated with Tris-EDTA Buffer (10mM, pH 6.0), overnight at 37°C. Retinas were blocked and permeabilised in a buffer containing 0.2% Tween20, 2% Triton X-100, 0.2% bovine serum albumin in PBS for 1 h at 20°C with gentle shaking. Retinas were washed in PBS and co-stained for microglia (1:500, Iba1; Novachem) and astrocytes (1:500, GFAP; Abcam) (1:500), overnight at 4°C with gentle shaking. For secondary antibodies, retinas were incubated with donkey anti-goat IgG AlexaFluor 647 (1:1000, ThermoFisher) for 2 h at 20°C with gentle shaking, then washed with PBS and incubated with goat anti-rabbit IgG AlexaFluor 546 (1:500, ThermoFisher) under identical conditions. Retinas were mounted onto Poly-Lysine coated slides (Tranjan) with the vitreous side facing upwards.

Immunofluorescence-labelled retinal wholemounts were imaged at 20x magnification using the Dragonfly Confocal

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Microscope (Andor, Oxford Instruments). Fluorescence micrographs were captured with the following settings for each excitation wavelength at: Iba1 – 594nm, 42.5ms exposure, 24% laser intensity; GFAP – 685nm, 42.5ms exposure, 18% laser intensity. Images were processed in FIJI software (ImageJ, US) and immunofluorescence intensities were analysed semi-quantitatively using Zeiss Zen Desk software.

2.8 Statistical analysis

Statistical analyses were made in GraphPad Prism 9 (U.S.A). Data were expressed as mean ± SEM and in all calculations, significance was regarded at $P< 0.05$. Distribution of the data was assessed using D'Agostino–Pearson omnibus normality test. The parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) for pairwise comparisons were used for normally distributed data. Pearson's coefficient was used to analyse the associations between OCT outcomes and biological and cognitive measures.

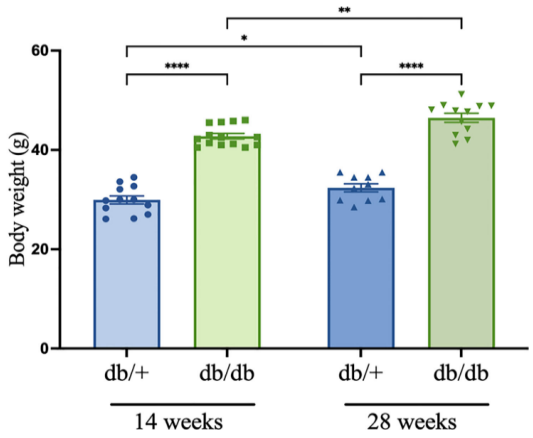

FIGURE 1
Body weight (g) measured prior to euthanasia in diabetic db/db and non-diabetic db/+ mice at 14 weeks and 28 weeks of age. Asterisks show statistical significance as *P<0.05, **P<0.01, ****P<0.0001. Animals weighed n = 8-12.

3 Results

3.1 Db/db mice exhibited an obese phenotype

The T2D obese phenotype was evident in the db/db model (Figure 1). By 14 weeks of age, body weight of db/db mice was 40% higher compared to control db/+ mice. At 28 weeks, body weight had significantly increased in db/db mice by 57% and 19% compared to db/+ mice and 14-week db/db mice, respectively.

3.2 Db/db mice display hyperinsulinemia, hypertriglyceridemia, and diabetic insulin resistance

We measured non-fasting levels of plasma glucose, insulin, and triglycerides in diabetic db/db and non-diabetic db/+ mice. Subsequently, the extent of insulin resistance was assessed with calculations of HOMA-IR and TyG index (Table 1). Plasma glucose levels in db/db mice were approximately twice as high compared to age-matched control db/+ mice at 14 weeks and 28 weeks of age. There was a 30% difference in plasma glucose levels between db/db mice across the experimental groups. Diabetic hyperinsulinaemia was evident at 14 weeks of age, with significantly elevated insulin levels in db/db mice compared to db/+ mice. Furthermore, plasma insulin was significantly elevated in 28-week db/db mice compared to db/+ mice and 14-week db/db mice. Triglyceride levels were increased by 13% in 14-week db/db mice and 8% in 28-week db/db mice compared to their age-matched db/+ controls, however the results were not statistically significant. The results demonstrated insulin resistance with significantly elevated HOMA-IR in 14-week db/db mice compared to control db/+ mice. The elevation in HOMA-IR was greater in diabetic mice at 28 weeks of age, with a 500% increase relative to control db/+ mice. There was a significant 90% increase in HOMA-IR in diabetic db/db from 14 weeks of age to 28 weeks of age. Insulin resistance was confirmed with significantly elevated TyG index in db/db mice compared to age-matched db/+ mice at 14 weeks and 28 weeks of age.

TABLE 1 Blood glucose, plasma insulin, triglycerides, HOMA-IR and TyG index.

	14 weeks		28 weeks	
	db/+	db/db	db/+	db/db
Glucose (mg/dL)	275 ± 39.2	625 ± 61.8**	221 ± 36.4	438 ± 64.3**,[a]
Insulin (µg/L)	1.48 ± 0.139	2.71 ± 0.584*	2.47 ± 0.392	5.64 ± 0.208***,[a]
Triglycerides (mmol/L)	0.325 ± 0.045	0.275 ± 0.037	0.241 ± 0.032	0.364 ± 0.077
HOMA-IR	22.4 ± 3.52	78.8 ± 18.3*	19.8 ± 5.98	145 ± 23.6***,[a]
TyG index	6.63 ± 0.271	7.54 ± 0.339*	6.09 ± 0.138	7.32 ± 0.258**

HOMA-IR, Homeostatic Model Assessment Index—Insulin Resistance; TyG index, Triglyceride-glucose index. Values presented as Mean ± SEM. Asterisk indicates statistical significance compared to age-matched db/+ mice by post-hoc Fisher's LSD test (*P<0.05, *P<0.05, **P<0.01, ***P<0.001). Superscript [(a)] notes statistical significance compared to 14-week db/db irrespective of degree of significance. Animals used for measures above n = 4-9.

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3.3 Diabetes was linked to reduction in thickness of the inner retina

Retinal OCT imaging, as represented in Figures 2A–E, revealed both diabetes-associated and age-related thinning in the mice. The measures of total neuroretina thickness were comparable in the central and mid-peripheral retina between diabetic db/db and non-diabetic db/+ controls at 14 weeks and 28 weeks of age (Figures 2F, G). There was a significant age-dependent reduction of the total neuroretina in the central retinal region by over 7 µm (2.5%) in both diabetic db/db mice and non-diabetic db/+ controls.

Among the sublayers, the GCC in both central and mid-peripheral retinal regions was of similar thickness in diabetic db/db mice and aged-matched control db/+ mice at 14 weeks of age (Figures 2H, I). At 28 weeks, the mean GCC thickness in db/db mice was significantly reduced by an average of 8 µm (10%) compared with db/+ controls. Furthermore, the GCC in both central and mid-peripheral regions showed significant reduction by over 9 µm (7.5%) in diabetic db/db mice from 14 weeks to 28 weeks of age.

Measurement of INL thickness revealed comparable results between db/db and db/+ animals in both central and mid-peripheral retina at 14 weeks and 28 weeks of age (Figures 2J, K). Compared to 14-week non-diabetic db/+ and diabetic db/db mice, INL measurement in the central retina of 28-week db/db and db/+ mice was decreased by 2 µm (8%) and 3.5 µm (11%), respectively.

Similar to the INL, ONL thickness was comparable between db/db and db/+ mice at 14 weeks of age (Figures 2L, M). Significant age-related ONL thinning was observed in diabetic db/db mice by 6 µm (9%) and non-diabetic db/+ mice by 7 µm (10.5%) from 14 weeks of age to 28 weeks of age. However, the age-dependent ONL thinning was less evident in db/db mice, showing significantly thicker ONL at 28 weeks by an average of 4 µm (6%), compared to age-matched db/+ mice.

Taken together, T2D was linked to changes in retina ultrastructure across various layers; age-related changes were

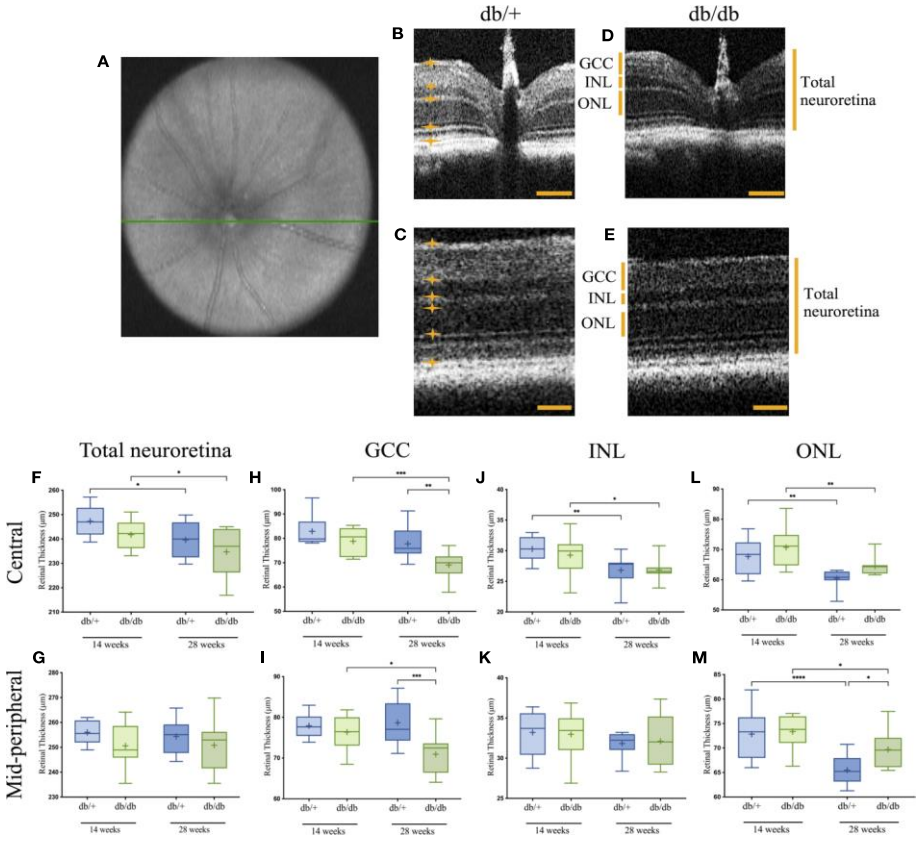

FIGURE 2
Representative OCT image of the central en-face scan with the optic nerve head indicated by a green line **(A)**, and B-scans of the central and mid-peripheral retinal regionals showing segmentation of the relevant layers in non-diabetic db/+ **(B, C)** and diabetic db/db **(D, E)** mice at 28 weeks of age. Six measurements were taken across each B-scan, with the boundary of each layer indicated by orange lines and asterisks. Scale bar is 50 µm. Boxplot graphs show data range with line through the median, and mean retinal thickness indicated as (+) for the GCC **(F, G)**, INL **(H, I)**, ONL **(J, K)**, and total neuroretina **(L, M)** in central (upper graphs). and mid-peripheral (lower graphs) regions with statistical significance noted as follows: **P<0.05, **P<0.01, ***P<0.001, ****P<0.0001*. GCC, ganglion cell complex; INL, inner nuclear layer; ONL, outer nuclear layer. Animals used for OCT imaging analysis, with the right eye included in the analysis n = 7-12.

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observed in the INL, ONL and total neuroretina. The most consistent finding from 2 sample regions of the retina was the decrease in thickness of the GCC with diabetes progression.

3.4 Diabetic mice exhibited cognitive decline in short- and long-term memory tests

To evaluate the impacts of diabetes on cognitive performance, mice underwent assessment of short-term and long-term memory using the novel object recognition (NOR) and passive avoidance (PA) tests, respectively (Figure 3). At 14 weeks of age, diabetic db/db mice exhibited intact short-term spatial learning and memory recall indicated by comparable Preference Index scores to non-diabetic control db/+ mice in the NOR test (Figure 3A). The Preference Index in db/db mice was significantly reduced compared to db/+ controls at 28 weeks of age, indicating substantial short-term memory deficits. The Preference Index was significantly lower in 28-week db/db mice compared to 14-week db/db mice, whilst the Preference Index in control db/+ mice showed no progressive deterioration of short-term memory.

In the PA test, diabetic db/db mice at 14 weeks of age showed significantly decreased latencies compared to age-matched non-diabetic db/+ mice, indicating long-term memory dysfunction (Figure 3B). The decrease in latency time was greater in 28-week db/db mice, also suggesting progressive loss of long-term memory.

3.5 Diabetic mice showed BBB breakdown

Cerebrovascular BBB integrity was examined using high throughput, automated quantitation of parenchymal extravasated plasma IgG (Figures 4A–C). At 14 weeks of age, db/db mice showed a non-significant 7-fold increase of IgG extravasation in the cerebral cortex compared to db/+ controls. Perivascular IgG extravasation in the hippocampus was comparable between db/db mice and db/+ controls 14 weeks of age. BBB dysfunction significantly increased with diabetic progression in db/db mice at 28 weeks of age, showing significantly elevated cortical and hippocampal IgG extravasation compared to age-matched db/+ controls and 14-week db/db mice (Figures 4B, C).

3.6 Diabetes induced heightened neuroinflammation and oxidative stress

Cerebral neuroinflammation and astrocytic activation were assessed *via* semi-quantitative intensity analyses of immunolabelled Iba1-positive microglia and GFAP-stained astrocytes, respectively. Oxidative DNA damage was evaluated using 8-OHdG immunoreactivity (Figure 5). Representative images are indicated in Figure 5A. At 14 weeks of age, db/db mice showed an average increase of 175% in Iba1 intensity compared to db/+ mice, although the difference did not reach significance (Figures 5B, C). There was a marked increase in microglial activation at 28 weeks, with significantly elevated Iba1 intensity confirmed in db/db mice relative to age-matched db/+ controls and the 14-week db/db group.

Reactive astrogliosis was prominent in the hippocampus of diabetic animals (Figures 5D, E). Indeed, db/db mice exhibited significantly elevated hippocampal GFAP intensity at 14 weeks compared to age-matched db/+ mice.

Quantitative analysis of 8-OHdG immunostaining revealed significant DNA oxidation in the cortex and hippocampus of 14-week db/db mice relative to db/+ mice (Figures 5F, G). Heightened oxidative stress persisted in db/db mice at 28 weeks with significantly greater 8-OHdG intensity in the hippocampus compared to db/+ mice. The 8-OHdG in the cortex of 28-week db/db mice was significantly reduced relative to 14-week db/db mice.

3.7 Diabetes increased retinal neuroinflammation

Microglial activation and astrocyte reactivity in the retina were examined *via* immunofluorescence microscopy, as shown in the representative images in Figure 6A. In 14-week db/db mice, Ibal immunointensity was statically comparable with db/+ controls. Microglial activation increased in db/db mice, showing

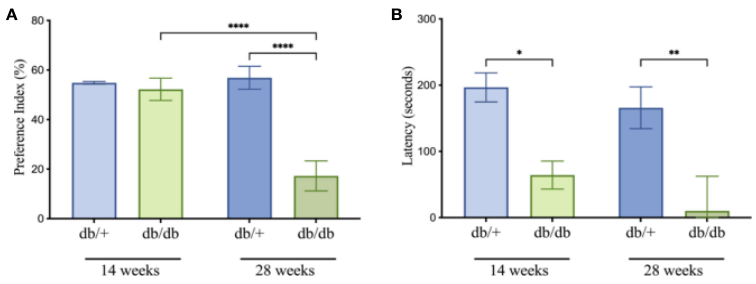

FIGURE 3
Cognitive testing for db/db and db/+ mice revealed deficits in the novel object recognition **(A)** and passive avoidance **(B)** tests. Statistical significance indicated as: **P<0.05, **P<0.01, and ****P<0.0001*. Animals used for cognition measures n= 8-12.

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significantly elevated Iba1 intensity in 28-week db/db mice compared to age-matched non-diabetic db/+ mice as well as 14-week db/db mice (Figure 6B). GFAP intensity in db/db mouse retina showed a modest 70% increase at both 14 and 28 weeks of age, compared to db/+ control mice (Figure 6C).

3.8 The retinal thinning in diabetes showed moderate correlations with cognitive decline disruption, neuroinflammation and oxidative stress

Pearson's correlation coefficient analysis revealed significant associations between retinal thickness and cognitive decline in diabetes (Figure 7). The total neuroretina thickness had a moderate positive correlation with performance in the PA test, but no apparent correlation with short-term recognition memory in the NOR test. Among the examined neural sublayers, GCC thinning in both central and mid-peripheral regions consistently correlated with short-term and long-term memory impairment in PA and NOR tests. In contrast, the relationship between INL thickness and cognitive performance varied from negligible to weak negative associations between INL (mid-peripheral) and Performance Index in the NOR test. There was a weak negative correlation between ONL thickness and cognitive outcomes.

Mixed results were found in the correlation assessment between retinal thickness and BBB permeability, as determined by IgG extravasation. The total neuroretina thickness in the central and mid-peripheral regions showed moderate negative correlations with IgG extravasation in the cortex; however, the relationship was reversed for the hippocampal formation. This trend was also observed in the sublayers, with GCC thickness in both regions of the retina negatively correlated with IgG extravasation only the cortex. There was a negligible to weak positive correlation between GCC thickness and IgG extravasation in the hippocampal formation. Conversely, INL thinning in the central, but not mid-peripheral, retina was linked to elevated IgG extravasation in the hippocampus. The ONL thickness at the central retina showed no correlation with global IgG extravasation, however, the mid-peripheral retina was positively correlated with hippocampal BBB permeability.

We also observed variable correlations between retinal thickness and neuroinflammation, measured as Iba1 intensity, in the cortex and hippocampal formation. The thickness of the total neuroretina and GCC showed weak to moderate negative correlations with Iba1 intensity, whereas the INL and ONL had weak to moderate positive correlations to neuroinflammation.

Furthermore, the total neuroretina thickness showed variable correlations to GFAP intensity, the principal measure of astrocyte reactivity. There was a moderate negative correlation with cortical GFAP, and a weaker negative correlation to hippocampal GFAP intensity. Within the retinal sublayers, the GCC thickness had negligible correlations with GFAP-positive astrocyte reactivity. Other notable correlations were between INL and ONL thickness in the central retina and GFAP intensity in the hippocampus, which showed contrasting moderate negative and positive associations, respectively.

Similarly, the marker of oxidative stress, 8OHdG in the cortex and hippocampus showed varied correlations with retinal thickness. The total neuroretina thickness in the mid-peripheral retina had a moderate negative correlation with cortical and hippocampal oxidative stress. In contrast, total neuroretina thickness in the central retina had weak negative and negligible associations to 8OHdG intensity in the cortex and hippocampus, respectively. The GCC and INL thickness had no correlation, whereas ONL thickness had a moderate positive correlation to oxidative stress.

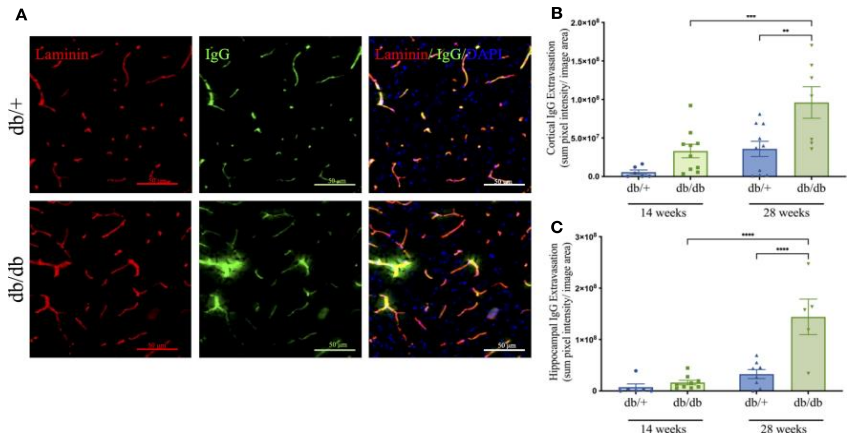

FIGURE 4
The blood-brain barrier integrity was assessed by immunostaining the basement membrane with laminin α4 (red) then detecting IgG extravasation (green) outside the capillary boundary, as shown in the representative images taken from the cerebral cortex of non-diabetic db/+ and diabetic db/db mice at 28 weeks of age **(A)**. Image scale bar is 50 µm. Mice at 14 weeks and 28 weeks of age were examined for parenchymal immunofluorescent IgG in the cerebral cortex **(B)** and hippocampus **(C)**. Statistical significance bars shown as ***P<0.01, ***P<0.001, ****P<0.0001.* Animals used for brain IgG analysis n= 6-10.

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Lastly, the relationships between retinal thickness and biomarkers of inflammation and gliosis were examined in the present study. The total neuroretina thickness showed no meaningful correlations with either retinal Iba1 or GFAP measures. In contrast, significant correlations were observed between GCC thinning and retinal neuroinflammation as well as astrocyte activation. Furthermore, the INL and ONL thickness showed weak correlations with Iba1-positive microglial and GFAP-labelled astrocytic activations, except for ONL (mid-peripheral) that had a weak negative relationship to retinal GFAP intensity.

Taken together, we observed that thinning in the total neuroretina and GCC was associated with elevated neuroinflammation, astrocyte reactivity, oxidative stress, and BBB permeability, apart from hippocampal IgG extravasation. Additionally, the total neuroretina and GCC layers showed the strongest relationship with memory performance in the PA and NOR tests. Conversely, ONL thickness had a reverse relationship to the total neuroretina and GCC pairwise comparisons.

4 Discussion

The metabolic sequalae of T2D, characterised by hyperglycaemia, insulin resistance, aberrant lipid metabolism and obesity, are evident in the db/db mouse model. As previously reported (18), diabetic mice in the present study exhibited a phenotype of obesity, elevated plasma glucose, a modest increase in plasma triglycerides, and decreased insulin sensitivity. These features of metabolic syndrome were present at 14 weeks of age and tended to worsen with diabetic duration. Specifically, the HOMA-IR and TyG index confirmed progressive insulin resistance in diabetic db/db mice at 28 weeks of age. Although HOMA-IR is widely utilised in clinical (19, 20) and preclinical diabetic literature (3, 21), TyG index is a relatively novel and inexpensive surrogate marker for insulin resistance that is positively correlated with HOMA-IR and glycated haemoglobin (HbA1c) (22, 23). Moreover, the TyG index shows promise in identifying individuals with T2DM-associated microvascular complications (24).

The present study investigated whether diabetic-induced changes in retinal thickness measured with *in vivo* OCT imaging co-occur

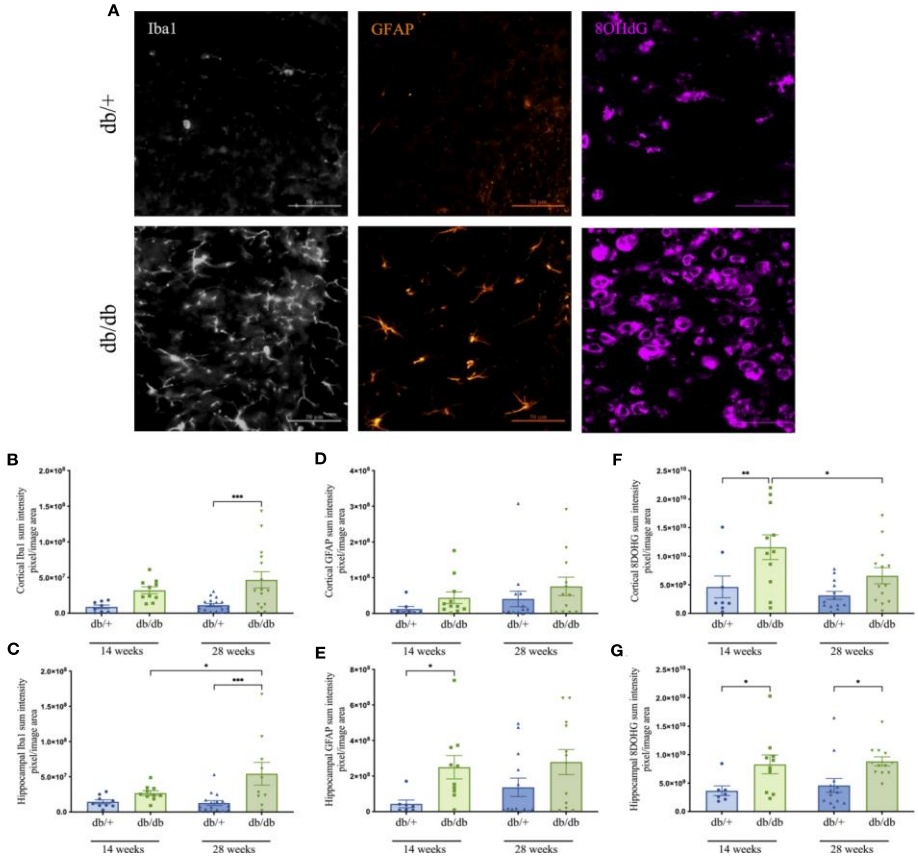

FIGURE 5
Representative images of Iba1- positive microglia in grey, GFAP- positive astrocytes in orange, and 8-OHdG cells that indicate DNA oxidation in violet captured within the cerebral cortex of non-diabetic db/+ and diabetic db/db mice at 28 weeks of age **(A)**. Image scale bar is 50 µm. Graphs show the immunointensities of Iba1 **(B, C)**, GFAP **(D, E)**, and 8-OHdG **(F, G)** in the cortex and hippocampus of db/db and db/+ mice at 14 weeks and 28 weeks. Statistical significance shown as **P<0.05, **P<0.01, ***P<0.001*. Animals used for brain immunofluorescence measures n= 7-12.

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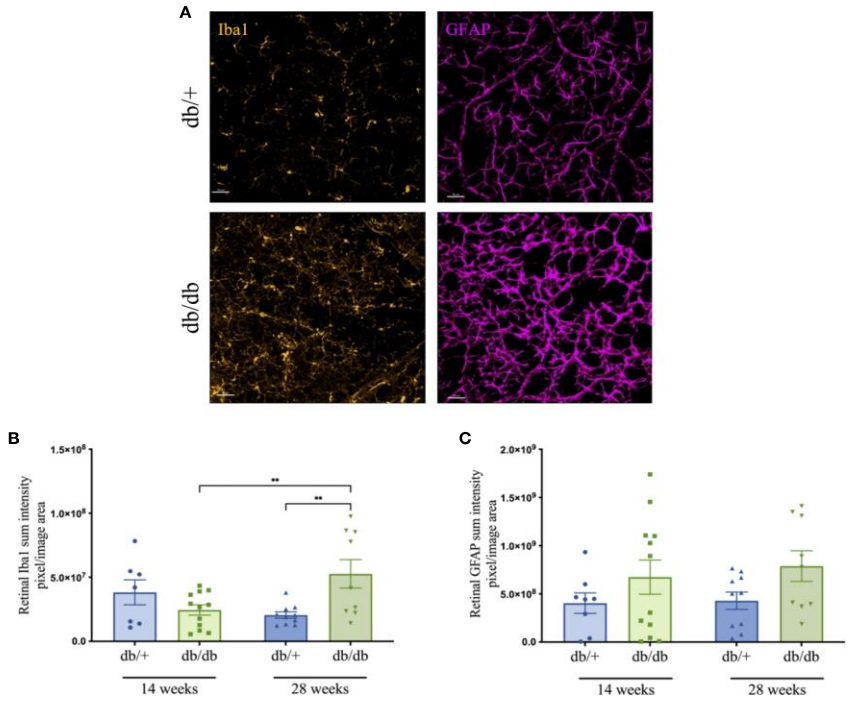

FIGURE 6
Representative immunofluorescence images (Iba1-positive microglia in orange and GFAP-positive astrocytes in violet) from the mid-peripheral region of retinal whole-mounts of non-diabetic db/+ and diabetic db/db mice at 28 weeks of age **(A)**. Scale bar is 50 µm. Changes in retinal Iba1 **(B)** and GFAP **(C)** immunointensity in db/+ and db/db mice at 14 weeks and 28 weeks of age. Statistical significance indicated as ***P<0.01*. Animals used for retinal immunofluorescence measures n= 8-12.

with cognitive decline and its underlying pathophysiology, notably neuroinflammation, oxidative stress and BBB hyperpermeability measured by *ex vivo* immunomicroscopy. Our OCT analyses revealed that the thickness of the total neuroretina and sublayers GCC and INL were progressively reduced in diabetic mice. Our findings were consistent with previous reports of ganglion cell loss in humans (25) and preclinical models of T2D that include db/db mice (10, 26–28). Indeed, diabetes associated neurodegeneration is most often identified in the innermost retinal layers that comprise the GCC: 1) the retinal nerve fibre layer (RNFL) that contains axons of ganglion cells and forms the optic nerve, 2) the ganglion cell layer (GCL) that includes the cell bodies of retinal ganglion cells (RGCs), and 3) the inner plexiform layer (IPL) that houses the RGC dendrites and their synapses with other cells of the retina (10, 29, 30). The mechanisms underlying neurodegeneration are still under investigation; however, mounting evidence suggests that neurons in the GCC are highly susceptible to apoptosis under diabetic pro-inflammatory and oxidative stress conditions (27). The latter is consistent with our observations showing significant progressive increase of neuroinflammation and suggestive astrogliosis in the retina of db/db mice. Interestingly in the present study, ONL thickness was increased in diabetic db/db mice at 28 weeks of age. This finding is consistent with some reports that the nuclear layers increase in thickness with diabetes (30, 31).

Concomitant with the changes in retinal thickness, diabetic db/db mice exhibited cognitive decline, with impairments in cortical-hippocampal dependent short-term and hippocampal-amygdala mediated long-term memory tests (32, 33). Similar impairments have been published in preclinical diabetes literature (4, 34, 35) using Morris water maze (MWM) and Barnes maze as measures of spatial learning, memory and cognitive flexibility, and y-maze as a marker of working memory (33, 36). Cognitive decline in diabetes is supported by neuroimaging data that show altered activity in brain regions involved in cognition (8).

Whilst the underlying mechanisms by which diabetes induces cognitive decline are largely unknown, existing research indicates cerebral microvascular abnormalities are a critical pathophysiology of diabetic cognitive decline (35). The BBB is composed of non-fenestrated capillaries with closely joined endothelial cells (due to junction proteins), atop an extracellular matrix basement membrane interspersed with pericytes (37). Astrocytic end-feet encircle these microvessels. Additionally, these astrocytes project towards adjacent neurons, forming close synaptic connections. Microglia monitor the perivascular microenvironment where they mediate debris phagocytosis, detection of harmful stimuli and neuroprotection through release of anti-inflammatory markers during acute inflammatory states. Together, these cells constitute the neurovascular unit (NVU), and are crucial for maintaining

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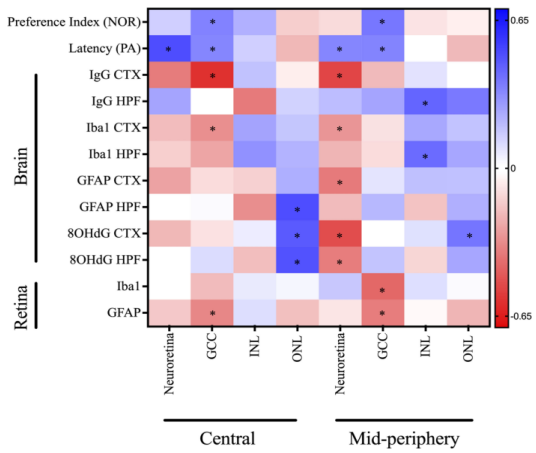

FIGURE 7
Correlation matrix of OCT measures against cognitive and immunofluorescence markers for neuroinflammation, oxidative stress, and BBB dysfunction depicted as a heatmap (corresponding colour scale provided and colour of each is cell is proportional to the value of the Pearson's correlation coefficient). The notable correlations that either neared or reached statistical significance of *P<0.05 include: Neuroretina central: Latency (r = 0.50; P=0.003), GCC central: Preference Index (r = 0.35; P=0.06), GCC central: Latency (r = 0.34; P=0.05), GCC central: IgG CTX (r = -0.59; P=0.03), GCC central: Iba1 CTX (r = -0.32; P=0.12), GCC central: GFAP retina (r = -0.34; P=0.06), INL central: GFAP HPF (r = -0.33; P=0.15), ONL central: GFAP HPF (r = 0.50; P=0.02), ONL central: 8OHdG CTX (r = 0.47; P=0.02), ONL central: 8OHdG HPF (r = 0.49; P=0.02), Neuroretina mid-peripheral: Latency (r = 0.34; P=0.05), Neuroretina mid-peripheral: IgG CTX (r = -0.53; P=0.05), Neuroretina mid-peripheral: Iba1 CTX (r = -0.30; P=0.14), Neuroretina mid-peripheral: GFAP CTX (r = -0.38; P=0.07), Neuroretina mid-peripheral: 8OHdG CTX (r = -0.51; P=0.01), Neuroretina mid-peripheral: 8OHdG HPF (r = -0.37; P=0.01), GCC mid-peripheral: Preference Index (r = 0.38; P=0.04), GCC mid-peripheral: Latency (r = 0.35; P=0.05), GCC mid-peripheral: Iba1 retina (r = -0.43; P=0.01), GCC mid-peripheral: GFAP retina (r = -0.37; P=0.04), INL mid-peripheral: IgG HPF (r = 0.44; P=0.14), INL mid-peripheral: Iba1 HPF (r = 0.42; P=0.05), and ONL mid-peripheral: 8OHdG CTX (r = 0.39; P=0.06). CTX, cortex; GCC, ganglion cell complex; HPF, hippocampal formation; INL, inner nuclear layer; NOR, novel object recognition; ONL, outer nuclear layer; PA, passive avoidance.

appropriate neurological function (38). In a dietary-induced model of insulin resistance, the disruption of the BBB was demonstrated to causally associate with cognitive decline (3). BBB dysfunction is reported to chronically lead to heightened neuroinflammation and oxidative stress, resulting in neurodegeneration and cognitive decline (3, 21). Consistently, the current study showed progressive BBB hyperpermeability in diabetic db/db mice concomitant with neurocognitive decline. In parallel, significant neuroinflammation, indicated by microglial activation and astrogliosis, and elevated oxidative stress were observed in the hippocampal formation and cortex of db/db mice. The substantial neuroinflammation in the db/db retina was comparable to cerebral inflammation, suggesting that neural tissue dysfunction is linked to diabetic pro-inflammatory pathways.

Neurodegeneration, an inflammatory and oxidative stress-mediated process leading to neuronal loss through up-regulated apoptotic signalling, is central to the pathophysiology of T2D (39–41). Chronic activation of Iba1-microglia and GFAP-astrocytes observed in the present study leads to the release of pro-inflammatory cytokines, chemokines, and adhesion factors, exacerbating glial activation and attracting immune cells and macromolecules such as immunoglobulins from the plasma (42). In this context, reactive astrocytes no longer mediate cellular metabolism, synaptic glutamate clearance, and neurovascular coupling (43, 44). The latter pathogenic event reflects the essential role of astrocytes in autoregulation, which is defined as the adjustment of blood flow to meet energy demands. Neurovascular disruptions are reported in diabetes (38, 45) and neurodegenerative conditions where cognitive impairment is a major symptom (41, 46). Glial over-activation also triggers increased production of free radicals, toxic metabolites, nitric oxide (NO), and pro-angiogenic factors, impairing BBB integrity, leading to ischemic lesions and atrophy observed in T2D models and patients (47, 48). The subsequent neuronal and vascular loss disrupts neural plasticity and functional connectivity, essential cognitive mechanisms.

Diabetic-induced inflammatory and oxidative stress responses observed in the retina parallel those in the brain (49). While MRI scans show alterations in brain structure (8, 50), OCT imaging allows cost-effective examination of retinal neurodegeneration (51). Reductions in the inner retina thickness correspond to functional alterations detected with multifocal electroretinography (mfERG) (52), and damage to the superficial vascular plexus can be observed with OCT angiography (39), even in the absence of clinical microvascular pathology. Therefore, it is likely that any potential links between GCC thinning and memory impairment may be attributed to similar patterns of diabetic neurodegeneration in both tissues.

We examined the association between *in vivo* OCT measures of retinal thickness and short-term and long-term memory, as well as brain and retinal neuroinflammation, oxidative stress, and BBB dysfunction. Pearson's correlation analysis revealed significant positive associations between retinal thickness and cognitive performance. In particular, thinning of the total neuroretina in the central and mid-peripheral regions was moderately correlated with impairment in long-term memory. Furthermore, GCC thickness in both retinal regions showed moderate positive associations with impairments in both short-term and long-term memory. Consistent with our findings, Allen et al. (53), reported that retinal thinning is concurrent with deficits in working memory and exploratory behaviour in the Goto-Kakizaki (GK) rat model of spontaneous T2D. Conversely, a thinner ONL layer was correlated with better cognitive performance, which is consistent with diabetic mice showing increased ONL thickness (30). The association between retina thickness and cognitive performance suggests that OCT imaging data may be used to identify elevated risk of cognitive decline in patients with T2D. This is supported by growing clinical evidence that confirms the link between diabetic retinopathy and cognitive decline, as reported in older people with T2D (54). The large-scale ACCORD trial by Hugenschmidt et al. (11), also reports cross-sectional and longitudinal correlations between retinopathy and diabetic cognitive decline, although changes in retinal thickness were not investigated.

The present data revealed significant associations between retinal thickness and cerebral BBB disruptions. Specifically, thinning of the total neuroretina and GCC in the central region were strongly associated with the cortical BBB disruption, whilst thinning of the INL was associated with elevated hippocampal BBB permeability. Upon examination of the mid-peripheral retina, there was an even stronger relationship between total neuroretina thinning and BBB disruption in the cortex. However, thickening of all retinal layers in the mid-peripheral region was associated with increased BBB permeability in the hippocampal formation. The underlying mechanisms for this regional variability remain obscure. Although IgG extravasation in the cortex and retinal ganglion cell layer has been shown to accompany retina thinning in diabetic pigs (55), no studies have yet correlated retinal structural changes to BBB hyperpermeability. Therefore, further research is necessary to explore the relationship between OCT retinal imaging and BBB integrity.

Our data also showed significant associations between changes in retinal thickness and brain pathophysiologies. The correlations between microglial activation and OCT retinal thickness were consistent across both retinal and brain regions, whereby total neuroretina and GCC thinning showed moderate associations with elevated microgliosis in the cortex; and the relationship was inversed for INL and ONL thickness. Astrogliosis and oxidative stress in the cortex and hippocampal formation were consistently associated with thinning of the total neuroretina in the mid-peripheral region and thickening of the ONL in both retinal regions. This suggests that structural changes across two or more retinal layers may be used in concert to improve the predictive capacity for cerebral pathophysiology. However more studies are required to replicate these findings and examine the temporal changes in the respective layers.

Finally, our correlation analysis revealed that GCC thinning was correlated with heightened neuroinflammation and astrogliosis in the retina, which confirms understanding that diabetic neurodegeneration is driven by chronic inflammation. To the best of our knowledge, this is the first study to investigate the relationship between retina thickness and a panel of neurophysiological markers for cognitive decline in a T2D model.

This study has some limitations due to the absence of repeated imaging and cognitive testing, which impedes the assessment of the predictive value of *in vivo* OCT imaging techniques. The implementation of a longitudinal study model would offer valuable insights into the temporal sequence of observed diabetic pathologies. Furthermore, pathological features of the db/db model such as hypertension were not explored in the study. Future studies should expand the battery of cognitive tests to evaluate deficits across various domains and their association with retinal microstructure. Furthermore, researchers should employ markers of neuronal loss to validate neurodegeneration *ex vivo* and corroborate OCT findings.

In summary, the results of the correlation assessment suggest that non-invasive *in vivo* OCT imaging may be utilised to identify a risk association with cognitive decline, cerebral BBB dysfunction and neuroinflammation in diabetes. These findings may be instrumental in the clinical detection of T2D patients at increased risk for future cognitive impairment, or who may already be exhibiting subtle, often overlooked, cognitive decrements The results also highlight the importance of considering specific retinal sublayers when investigating the relationship between the retina and cognitive function in T2D. However, further studies with more robust data sets are needed to increase the statistical power of the correlations.

Data availability statement

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Ethics statement

The animal study was approved by Curtin Animal Ethics Committee, approval no. ARE 2018-19. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MM - Responsible for day-to-day running of the animal model, coordinating and assisting with OCT imaging, conducting ex vivo measures and data analysis, writing and editing manuscript. SM – Animal anesthesia, collection of OCT images and guidance on interpreting OCT imaging data, editing manuscript. MN – Assistance with sample collection and optimisation of immunofluorescence staining and analysis. PB – Preliminary literature review on OCT. FC – consultation and advice regarding suitability of OCT imaging in animal model. VL –assistance with data interpretation, manuscript editing. JM – Guidance on animal model, writing and editing manuscript. RT – oversight of project, expertise on animal model and study design, assistance with data interpretation, writing and editing manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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2.3 ANALYSIS OF PLASMA INFLAMMATORY MARKERS IN DB/DB MICE AND THEIR ASSOCIATION WITH DIABETIC COGNITIVE DYSFUNCTION

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2.3.1 Abstract

Background: Cognitive dysfunction and cerebral pathological changes are commonly reported in type 2 diabetes via unknown mechanisms. Type 2 diabetes involves low-grade chronic inflammation, represented by altered plasma pro-inflammatory cytokine profile. However, the relationship between these inflammatory changes and cognitive dysfunction, along with their underlying mechanisms, remains underexplored.

Methods: This study investigated the plasma levels of 13 inflammatory cytokines and chemokines using the BioLegend LEGENDplex kit (TNF-α, IL-1α, IL-1β, IL-6, IL-10, MCP-1, IFN-β, IFN-γ, IL-17, IL-23, IL-27, IL-12p70, and GM-CSF) in diabetic db/db mice and non-diabetic db/+ controls at 14 and 28 weeks of age. Subsequently, a Pearson's correlation analysis was performed to examine the relationship between inflammatory markers with diabetic alterations and pathophysiological markers of cognitive dysfunction. The CNS measures included short-term and long-term memory function, BBB leakage, astrocyte reactivity (GFAP), microglial activation (Iba1), and DNA oxidation (8OHdG) in the brain, as well as glial activation (GFAP and Iba1) in the retina.

Results: Diabetic db/db mice exhibited elevated plasma levels of TNF-α, IL-1α, IL-1β, IL-6, IL-10, and MCP-1, particularly at 14 weeks. Interestingly, there was a general decline in these cytokines by 28 weeks, a trend observed regardless of diabetic status. In the 28-week cohort, there was a slight increase in plasma concentrations of what? in db/db mice compared to non-diabetic db/+ controls, including the significant elevation of MCP-1. Furthermore, there were mixed associations between inflammatory markers and CNS pathophysiology in T2D. Notably, 7 inflammatory markers showed low detection rates or were completely undetectable in these mice.

Discussion: These findings indicate that while examining pro-inflammatory markers can provide valuable insights, greater assay sensitivity and a refined selection of inflammatory markers are necessary to robustly understand the diabetic changes and inflammatory status in these db/db mice. Additionally, the variable associations, particularly in neuroinflammation in the brain and retina, suggest that this set of cytokines/chemokines may not consistently serve as a reliable surrogate for CNS complications associated with cognitive decline in T2D.

2.3.2 Introduction

Type 2 diabetes is an established risk for cognitive decline and dementia. Whilst underlying mechanisms are largely unknown, an accumulating body of evidence suggests that the disruption of NVU and glial activation, induced by chronic inflammation may be pivotal.

Systemic inflammation significantly contributes to the development and progression of T2D and its associated complications (Galicia-Garcia et al., 2020). Elevated levels of pro-inflammatory mediators in the bloodstream contribute to vascular injury and oxidative stress, leading to breakdown of the NVU and glial activation in the CNS (Kaur et al., 2018; Piatkowska-Chmiel et al., 2021). Key cytokines and chemokines such as tumour necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), and interleukin-1 beta (IL-1β) are implicated in the pathogenesis of T2D and its effects on the brain and retina (Elmarakby & Sullivan, 2012; Sinclair et al., 2022). Other inflammatory mediators, including monocyte chemoattractant protein-1 (MCP-1), interferons (IFN- β and IFN-γ), and interleukin-1 alpha (IL-1α), are also involved in T2D-related complications (Du et al., 2023). However, the specific inflammatory profile in T2D is complex and continues to be investigated.

Despite the established roles of inflammatory markers in T2D, their associations with cognitive dysfunction remain inconsistent. Some studies have shown that elevated levels of TNF-α, IL-1β, and IL-6 are linked to worse neurophysiological performance in individuals with T2D (Du et al., 2023), while others have not observed this relationship (Dyer et al., 2020). Additionally, there is a lack of research comparing circulatory inflammatory markers with markers of CNS pathophysiology, including BBB leakage, glial activation and oxidative injury.

This study aims to quantify the concentration of 13 cytokines/chemokines in the plasma of diabetic db/db mice and non-diabetic db/+ controls at 14 and 28 weeks of age. Furthermore, the study aims to investigate the relationship of these inflammatory markers to cognitive dysfunction and its underlying pathophysiologies in the brain and retina tissue. We hypothesise that db/db mice will exhibit cytokine dysregulation, and the changes in plasma cytokines will have variable correlations with CNS pathology relating to cognitive dysfunction.

2.3.3 Methods

For animals, retinal imaging, short-/long-term memory assessments, and immunofluorescent microscopy please refer to the main manuscript of Chapter 2 of this thesis (Majimbi et al., 2023).

The plasma cytokine profile was determined using the LEGENDplex Mouse Inflammation Panel (13-plex) with V-bottom Plate (BioLegend Cat. No. 740446), which contains tumour necrosis factor alpha (TNF-α), interleukins (IL-1α, IL-1β, IL-6, IL-10, IL-17, IL-23, IL-27, and IL-12p70), monocyte chemoattractant protein-1 (MCP-1), interferons (IFN-β and IFN-γ), and granulocyte-macrophage colony-stimulating factor (GM-CSF). Gating strategy is represented in Supplementary 1.

A modified protocol, previously used in our lab was employed (D'Alonzo et al., 2020). Plasma samples from diabetic db/db mice and nondiabetic db/+ controls at 14 weeks and 28 weeks, collected and stored as per Chapter 2 manuscript, were prepared undiluted in a 96-well plate. For the assay setup, 10 µL of Matrix C and Assay Buffer were added to standard and sample wells, respectively, followed by 10 µL of standard or sample. Unique fluorescence-intensity cytokine beads were added to each well and incubated for 2 hours. Thereafter, the protocol included a centrifuge wash cycle, incubation with detection antibodies, addition of SA-PE, and a final wash prior to flow cytometric analysis using BD Fortessa Flow Cytometer (BD, San Jose, CA, USA). The results were processed using FlowJo (FlowJo, OR, USA).

Statistical analyses were conducted using GraphPad Prism 9 (USA). Data are presented as mean ± SEM, with significance set at $P < 0.05$. The D'Agostino-Pearson omnibus test was applied to assess data normality. For normally distributed data, a parametric one-way analysis of variance

(ANOVA) followed by Fisher's least significant difference (LSD) post-hoc test was used for pairwise comparisons.

Correlations were examined using Pearson's coefficient to assess the relationship between plasma inflammatory markers and the broader pathophysiology of diabetic cognitive dysfunction. Animals used in the correlation analysis include diabetic db/db mice and nondiabetic db/+ controls on standard chow at 14 weeks and 28 weeks of age.

2.3.4 Results

The broader CNS pathologies of diabetic db/db mice at 14 and 28 weeks of age have been published (Majimbi et al., 2023). Briefly, db/db mice exhibited metabolic disturbances at both experimental timepoints, with exacerbated insulin resistance and plasma triglycerides observed in the 28-week group. Additionally, db/db mice at 28 weeks demonstrated both short-term and long-term memory impairments and retinal degeneration, particularly affecting the GCC. Immunofluorescence microscopy revealed significant CNS pathophysiology in the cortex and hippocampus in db/db mice at 28 weeks, including BBB hyperpermeability, astrogliosis, chronic microglial activation, and oxidative DNA damage. The retina also exhibited glial pathology similar to cerebral neuroinflammation.

In the present study, inflammatory markers that did not reach detection levels are included in Supplementary 2, while those that reached sufficient detection levels are discussed below.

At 14 weeks, db/db mice demonstrated a significant increase in plasma TNF-α levels compared to db/+ controls, (Figure 2.9A). While the TNF-α concentrations in db/db mice at 28 weeks suggested a decline, this did not achieve statistical significance when compared to the levels of the 14-week db/db mice.

Regarding the pro-inflammatory interleukins, IL-1α, IL-1β, and IL-6 plasma concentrations were significantly higher in the db/db cohort relative to the db/+ controls at 14 weeks (Figures 2.9B-D). An age-associated downtrend in these cytokines' levels was observed in both experimental groups at 28 weeks, with only the decrease in IL-6 in db/db mice reaching statistical significance.

Although the cytokine levels appeared marginally increased in db/db mice at 28 weeks, these differences did not reach statistical significance. The substantial proportion of data below the detection threshold in the 28-week non-diabetic db/+ controls potentially confounded the statistical analyses.

For IL-10, a cytokine typically associated with anti-inflammatory responses, db/db mice exhibited a significant elevation at 14 weeks when compared with db/+ controls (Figure 2.9E). By 28 weeks, there was no discernible difference in IL-10 levels between the diabetic and non-diabetic groups. Noteworthy, however, was the significant reduction in IL-10 concentrations in db/db mice at 28 weeks compared to their 14-week counterparts.

MCP-1 concentrations were significantly higher in db/db mice at 14 weeks relative to non-diabetic db/+ controls and persisted to remain elevated at 28 weeks (Figure 1F). Yet, older db/db mice presented with significantly reduced MCP-1 levels in comparison to the younger db/db cohort at 14 weeks.

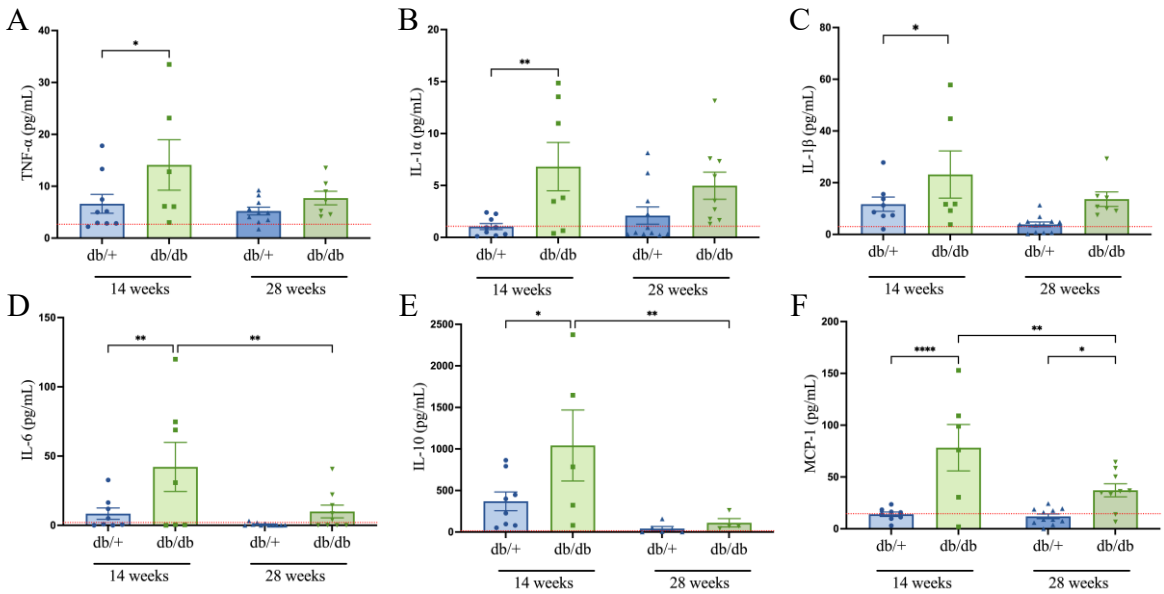

Figure 2-9 Graphs indicate plasma proinflammatory cytokine/chemokine concentrations in picograms per millilitre (pg/mL).

Levels of TNF-α, IL-1α, IL-1β, IL-6, IL-10, and MCP-1 (A-F) are measured in diabetic db/db mice and non-diabetic db/+ controls at 14 and 28 weeks of age. Results expressed as mean ± SEM (n = 4-10). Asterisks show statistical significance as *P<0.05, **P<0.01, ****P<0.0001.

The Pearson's correlation analysis revealed diverse associations between peripheral inflammatory mediators and cognitive dysregulation in T2D, as illustrated in Figure 2.10. TNF-α, IL-1α, IL-6, and MCP-1 showed weak to mild positive correlations with short-term memory function in the NOR test. However, their associations with long-term memory function in the PA test were less pronounced, indicating weak to negligible correlations. In contrast, IL-1β exhibited strong negative associations with both short-term and long-term memory performance, which were statistically significant, suggesting a potentially detrimental role in cognitive function. The anti-inflammatory cytokine IL-10 displayed a statistically significant strong positive correlation for short-term memory and a moderate negative correlation for long-term memory.

Regarding cerebral pathophysiological mechanisms, the associations between proinflammatory cytokines/chemokines and BBB leakage and astrocyte reactivity were mostly negligible or weak. However, there were moderate positive associations between cerebral microglial activation and plasma levels of pro-inflammatory markers (particularly TNF-α, IL-1β, and MCP-1). Interestingly, proinflammatory cytokines did not show meaningful correlations with DNA oxidation in the brain.

In the retina, the correlation between cytokines and astrocyte reactivity ranged from negligible to weak. Furthermore, plasma TNF-α, IL-1β, and IL-6 showed moderate to strong negative associations with microglial activation in the retina, contrasting with their associations in the brain.

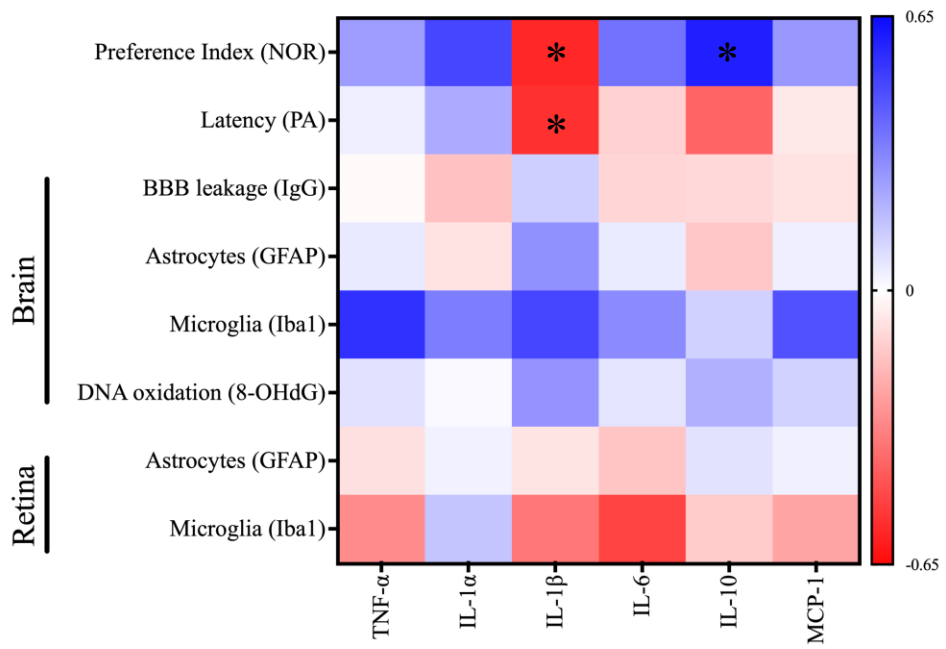

Figure 2-10 Correlation matrix of plasma proinflammatory cytokine against memory performance and immunofluorescence markers for BBB dysfunction, glial activation, and DNA oxidation.

The heatmap presents blue-red colour scale that corresponds to the value of the Pearson's correlation coefficient. Asterisks show statistical significance as *P<0.05.

2.3.5 Discussion

This study explored the systemic inflammatory profile in the db/db model of T2D and its correlation with cognitive dysfunction and markers of CNS pathophysiology. Our previous research in these animals demonstrated significant CNS complications, including memory impairments, inner retinal thinning, and associated BBB breakdown, glial activation and oxidative stress (Majimbi et al., 2023). This study offers an opportunity to further understand the pathological changes in the db/db model, and to compare the relationship of cognitive dysfunction to peripheral inflammatory markers and retinal neurodegenerative changes.

Our findings revealed significant elevations in pro-inflammatory mediators (TNF-α, IL-1α, IL-1β, IL-6, and MCP-1) in db/db mice at 14 weeks, corresponding to early T2D phenotypes, including metabolic dysregulation and obesity. This is in line with prior studies in both human and animal models of T2D that document a similar inflammatory profile (Galicia-Garcia et al., 2020).

Accumulating evidence suggests that T2D-related immune dysfunction and metabolic dysregulation promote one another, accelerating pancreatic dysfunction, insulin resistance and dyslipidaemia (McCoy & Tansey, 2008; Velikova et al., 2021). The observed increase in the anti-inflammatory cytokine IL-10 suggests a compensatory immunoprotective mechanism. However, even an upregulation of IL-10 can contribute to tissue degeneration and vascular damage by disrupting essential signalling pathways (Okdahl et al., 2022). Therefore, the altered cytokine profiles of db/db mice signal the escalation of diabetic phenotypes and associated complications (Donath & Shoelson, 2011).

The literature presents a complex web of interactions among plasma cytokines, where molecules like IL-6 and TNF-α can stimulate each other's production, while IL-10 typically acts to suppress IL-6 (Kassan et al., 2011). However, this anticipated inverse relationship between IL-10 and IL-6 was not mirrored in our data, as we observed concurrent elevation and reduction of both cytokines. Furthermore, while IL-6 is known to trigger MCP-1 production (Nirenjen et al., 2023), our study did not capture a distinct relationship beyond the broader trends noted for all inflammatory mediators. Future studies in the db/db model could explore the mechanistic interactions between cytokines and chemokines alongside plasma measurements.

A striking observation in this study was the overall reduction in plasma cytokines and chemokines from 14 to 28 weeks, especially in diabetic db/db mice. Interestingly, this decline was also seen, although to a lesser degree, in the nondiabetic db/+ control mice. This finding is surprising, as it contradicts the expected increase in inflammation typically associated with the progression of diabetes and the natural aging process (Li et al., 2023; Liu et al., 2016; Pan et al., 2021). It is noteworthy that concurrent CNS pathology in db/db mice increased with age and diabetes progression (Majimbi et al., 2023), despite the decrease in plasma inflammatory cytokines. This dissociation raises questions about the interplay between systemic inflammation and local CNS changes.

To explain the potential age-related suppression of the systemic inflammatory response, several factors may be at play. Earlier stages of diabetes might trigger strong inflammatory responses, but as the disease becomes chronic, the nature of the immune response can shift. In this experimental

cohort, hyperglycaemia was significantly higher at 14 weeks compared to 28 weeks in db/db mice, suggesting that the observed increase in inflammatory mediators may be a transient effect corresponding to the state of dysglycaemia (Galicia-Garcia et al., 2020). Additionally, the phenomenon of immunosenescence in T2D, signifying an acceleration of age-related deterioration of the immune system (Barbé-Tuana et al., 2020), might provide an explanation for the observed decrease in cytokine concentrations. The ongoing pathology in the CNS suggests that once certain thresholds of damage are crossed, they may not fully recover even if systemic inflammation subsides.

Our results from the Pearson's correlation analysis of the aggregated dataset (including 14- and 28-week timepoints) provide a nuanced perspective on the inflammatory mediators' role in T2D-associated CNS complications. The analysis showed that the elevation of plasma pro-inflammatory IL-1β is strongly associated with better short-term and long-term memory performance in diabetic db/db mice. Conversely, the elevation of anti-inflammatory IL-10 in plasma was associated with lower performance of short-term memory. Such findings are consistent with clinical studies that present both supporting and conflicting evidence regarding the associations between circulating pro-inflammatory cytokines and cognitive impairment in T2D (Du et al., 2023; Dyer et al., 2020).

For markers of cerebral pathophysiology, no correlations were observed between plasma cytokines and BBB leakage or astrocyte reactivity. Strong to moderate positive associations were suggested between microglial activation and the proinflammatory mediators (TNF-α, IL-1β, IL-6, and MCP-1). A positive relationship between inflammatory mediators and neuroinflammation, despite their divergent trajectories with age, suggests that systemic inflammation may influence or exacerbate CNS pathology in T2D, independent of the BBB disruption or astrocyte activation. These findings are supported by data linking inflammation and CNS injury (Liu et al., 2016; Pan et al., 2021). Interestingly, these cytokines did not correlate with DNA oxidation in the brain, suggesting separate mechanistic pathways for inflammation and oxidative stress within the T2D paradigm.

When examining the retina, the proinflammatory cytokines exhibited unique associations with glial activation compared to the brain. The retina is known to be susceptible to cytokine/chemokine-mediated neuroinflammation through the BRB and remote activation

pathways (Kuo et al., 2022). Despite glial activation being a prominent feature in both the brain and retina, serving as a shared pathophysiology of CNS complications in the db/db model (Majimbi et al., 2023), the variable weak to moderate correlations observed suggest that peripheral cytokines may not accurately reflect what is happening in both neural tissues. These findings underscore the importance of considering tissue-specific effects of cytokines when examining T2D complications.

This study indicates that changes in peripheral cytokine levels in T2D may not directly reflect CNS pathology, especially as the disease progresses. While plasma biomarkers are feasible and cost-effective, ongoing research should expand the range of peripheral markers studied and compare these with assessments of CNS-specific proinflammatory cytokines/chemokines. To strengthen these findings, a larger cohort of animals and longitudinal data from diabetes onset through various disease stages are needed to differentiate age-related effects from those specifically related to T2D. Moreover, a significant number of cytokines exhibited low detectability rates in this study, highlighting the need for assays with greater sensitivity. This may require an immunoassay panel capable of measuring smaller concentrations in plasma.
Although not explored in this thesis, evaluating the impact of antidiabetic drugs like metformin and anti-inflammatory agents like SAC and CBD on cytokine profiles could provide valuable insights into managing T2D-related inflammation.

In conclusion, the early T2D phenotype in the db/db mouse model was associated with heightened pro-inflammatory cytokines. A paradoxical decrease in peripheral inflammation was noted with age and diabetes duration. The correlations between inflammatory cytokines and CNS pathology appeared inconclusive. Considering these findings alongside our published work in this chapter, we endorse the use of GCC thickness as a more consistent predictor of cerebral pathology in T2D.

Acknowledgement

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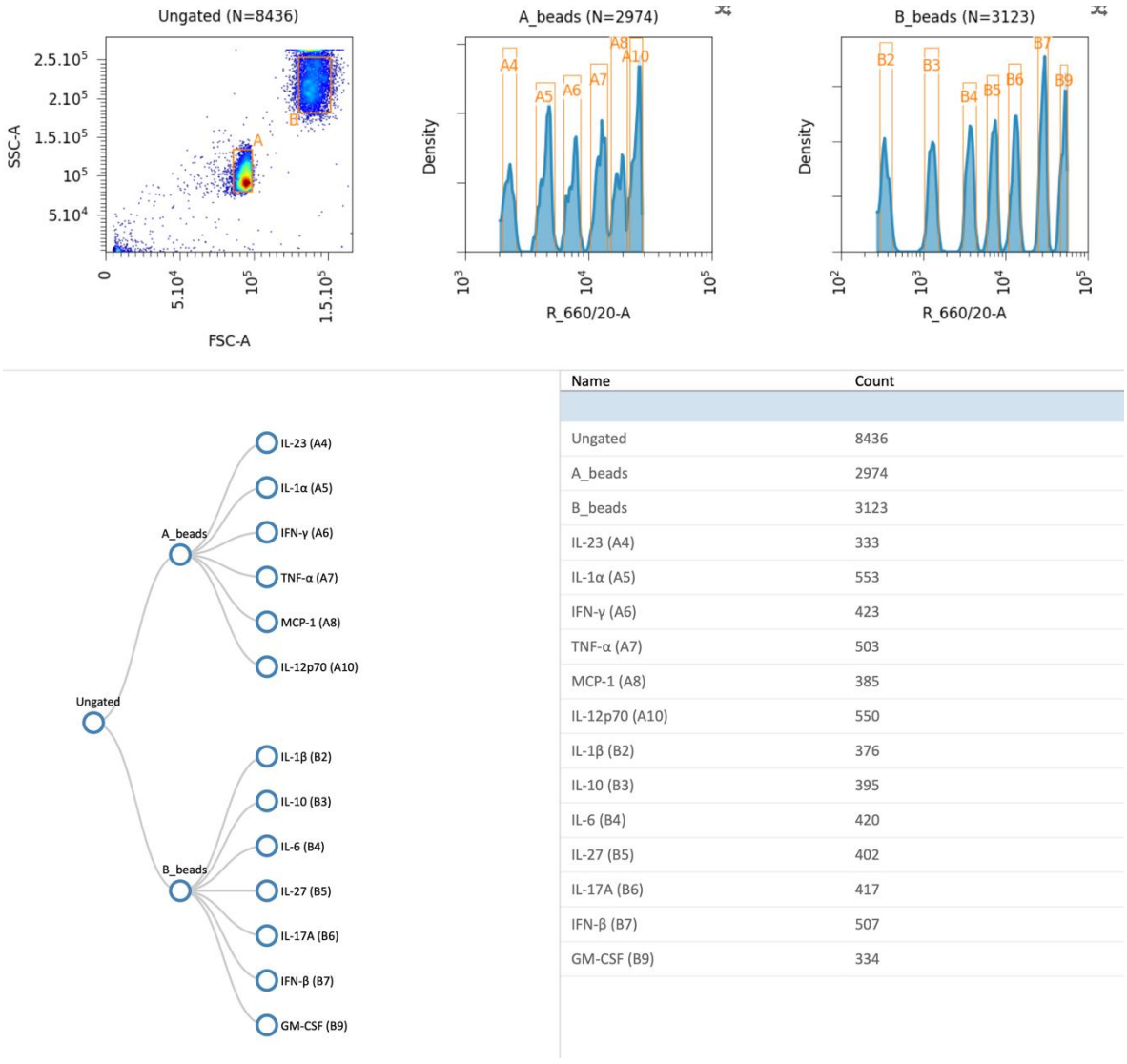
2.3.6 Supplementary 1



Name	Count
Ungated	8436
A_beads	2974
B_beads	3123
IL-23 (A4)	333
IL-1α (A5)	553
IFN-γ (A6)	423
TNF-α (A7)	503
MCP-1 (A8)	385
IL-12p70 (A10)	550
IL-1β (B2)	376
IL-10 (B3)	395
IL-6 (B4)	420
IL-27 (B5)	402
IL-17A (B6)	417
IFN-β (B7)	507
GM-CSF (B9)	334

Figure 2-11 Representative gating strategy for LEGENDplex Mouse Inflammation Panel (13-plex) with V-bottom Plate (BioLegend Cat. No. 740446).

2.3.7 Supplementary 2

Table 2-2 Cytokines that did not reach detection levels.
This table presents the mean plasma concentrations (pg/mL), standard error of the mean (SEM), and the percentage of samples above the limit of detection (LOD). Values are represented as mean ± SEM.

			14 weeks db/+	**14 weeks db/db**	**28 weeks db/+**	**28 weeks db/db**
IFN-γ (LOD: 0.81)	Mean		10.9	8.1	0.8	2.0
	SEM		5.0	2.8	0.2	0.8
	Percentage above LOD (%)		88.89	75.00	50.00	66.67
TNF-α (LOD: 2.66)	Mean		4.3	8.1	1.6	2.8
	SEM		1.6	3.8	0.4	0.8
	Percentage above LOD (%)		66.7	87.5	75.0	88.9
IFN-β (LOD: 130.84)	Mean		11.6	3.3	0.0	1.1
	SEM		7.8	1.7	0.0	0.6
	Percentage above LOD (%)		44.4	62.5	16.7	44.4
IL-12p70 (LOD: 11.03)	Mean		0.4	2.5	0.0	0.2
	SEM		0.3	1.3	0.0	0.1
	Percentage above LOD (%)		33.3	62.5	0.0	22.2
IL-27 (LOD: 20.24)	Mean		147.1	327.8	55.6	17.6
	SEM		62.9	179.8	19.7	5.0
	Percentage above LOD (%)		88.9	62.5	58.3	88.9
GM-CSF	Mean		3.6	7.0	0.0	1.0

(LOD: 15.04)	SEM		1.7	2.1	0.0	0.5
	Percentage above LOD (%)		55.6	75.0	0.0	33.3
	Mean		12.8	47.8	0.0	1.0
IL-23	SEM		6.4	28.4	0.0	0.5
(LOD: 42.3)	Percentage above LOD (%)		66.7	87.5	0.0	55.6
	Mean		7.3	44.7	0.0	1.8
IL-17A	SEM		4.1	18.7	0.0	1.0
(LOD: 8.66)	Percentage above LOD (%)		55.6	62.5	25.0	66.7

CHAPTER 3. NEUROPROTECTIVE THERAPIES IN T2D

3.1 SYNOPSIS

3.1.1 Background:

This chapter explores the neuroprotective potential of SAC and CBD compared to the conventional T2D medication, metformin, in maintaining cognitive function and retinal health in the diabetic db/db mouse model. Cognitive and retinal complications in T2D are linked to neurodegenerative disease mechanisms, including neurovascular compromise, glial-mediated inflammation, and oxidative stress (Chapter 2). SAC and CBD show promise in reducing inflammation and inflammation, suggesting therapeutic benefits in T2D-related CNS complications. However, the effects of long-term treatment and the potential for synergistic effects with SAC+CBD combinatory treatment are unknown. Leveraging the moderately diabetic phenotypes observed in Chapter 2, this study considered a preventative dietary intervention study for protection of CNS functions in T2D.

3.1.2 Methods in brief

This study assessed the therapeutic impact of SAC, CBD, SAC+CBD and metformin over 23 weeks of dietary treatment interventions in the LepR db/db mouse model (Figure 3.1). Animals were assessed for memory function, OCT-derived retinal thickness, and ex vivo immunofluorescence markers of CNS pathophysiologies as recently published (Majimbi et al., 2023), with the addition of BRB leakage to our suite of measures.

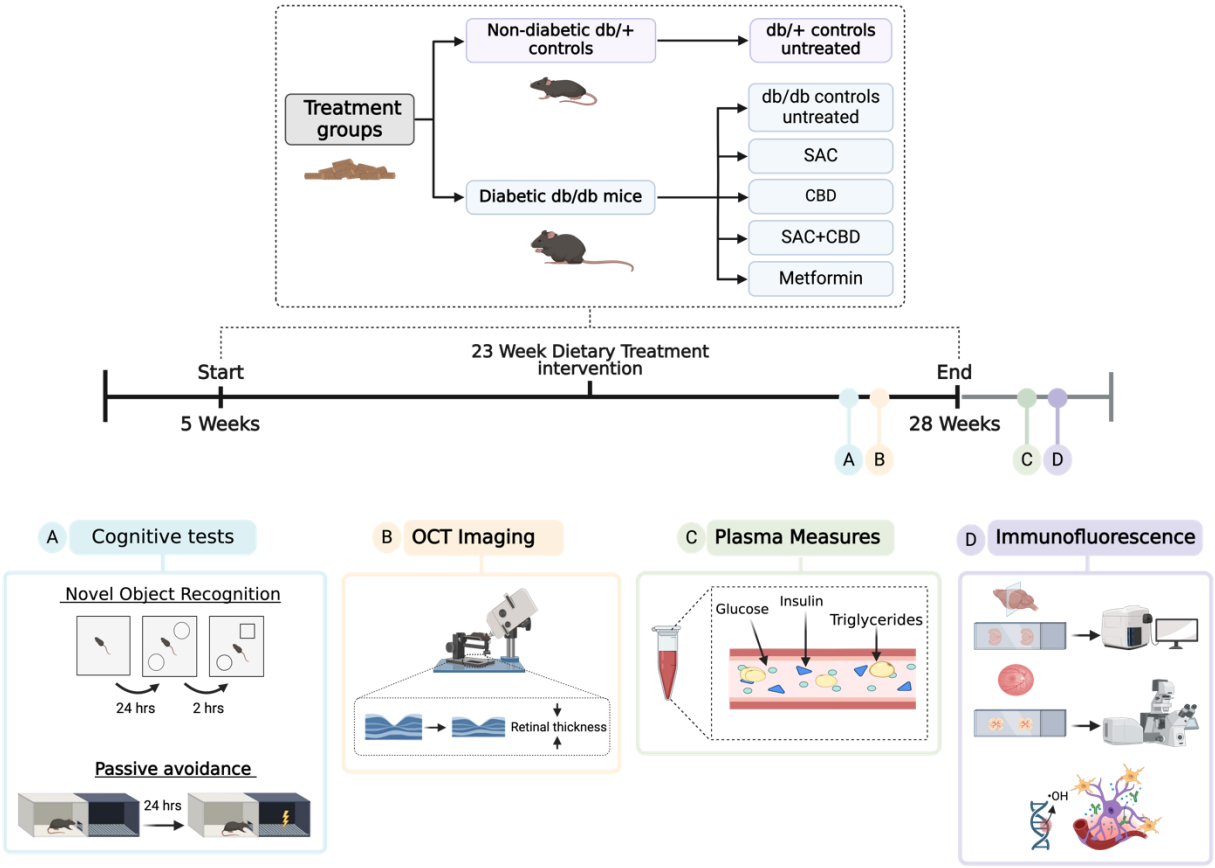


Figure 3-1 Research timeline for dietary intervention with SAC, CBD, SAC+CBD and metformin. *Made with biorender.*

Additionally, quantification of CBD in db/db mice following dietary treatment intervention study was carried out as described (Majimbi et al., 2021), and the findings have been discussed in the Chapter 3; Supplementary 1.

3.1.3 Results in brief

The results showed that SAC and CBD significantly improved CNS pathologies in db/db mice, diminishing glial activation and synergistically preventing BBB leakage. These compounds preserved retinal thickness and improved memory, performing comparably or superiorly to metformin against CNS pathologies associated with diabetes.

The follow-up study provides evidence for the bioavailability of CBD in the brain and plasma of db/db mice following chronic dietary administration.

3.1.4 Discussion in brief

While SAC and CBD did not significantly improve metabolic parameters, their pronounced effects on glial activation and oxidative stress, and synergistic neurovascular preservation point to their neuroprotective role. These effects potentially contribute to the observed preservation of memory functions and retinal health. Based on these findings, we posit that natural agents have therapeutic efficacy in treating CNS complications of T2D. We suggest that ongoing investigations, particularly in longitudinal test paradigms, are needed to elucidate the mechanistic and pharmacokinetics of SAC and CBD, alone and in combination, to build upon our findings.

The work presented in this chapter has been submitted to *Scientific Reports* for peer review but has been reformatted for inclusion in this thesis.

3.2 S-ALLYL CYSTEINE AND CANNABIDIOL ARE EQUALLY EFFECTIVE AS METFORMIN IN PRESERVING NEUROVASCULAR INTEGRITY, RETINAL STRUCTURE, AND COGNITIVE FUNCTION IN DB/DB TYPE 2 DIABETIC MICE

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3.2.1 Abstract

Type 2 diabetes mellitus (T2D) is associated with central nervous system (CNS) alterations marked by neurovascular, inflammatory, and oxidative damage, resulting in cognitive dysfunction and retinal neurodegeneration. This study explored the therapeutic potential of naturally derived S-allyl-cysteine (SAC) and cannabidiol (CBD) in LepR db/db mice, targeting blood-brain/retinal barrier (BBB/BRB) leakage, glial activation, and DNA oxidative damage to alleviate memory deficits and retinal neurodegeneration.

Diabetic mice underwent 23 weeks of dietary treatments: diabetic db/db controls, SAC, CBD, SAC+CBD, and metformin, with nondiabetic db/+ mice as negative controls. Memory function was assessed using novel object recognition and passive avoidance tests, while retinal thickness was measured via *in vivo* OCT imaging. Immunofluorescence imaging quantified neurovascular leakage (IgG extravasation), glial activation (GFAP and Iba1) and DNA oxidation (8OHdG) in brain sections and retinal whole-mounts.

In diabetic db/db mice with hallmark metabolic dysregulation, CNS alterations included BBB/BRB leakage, glial activation, and retinal neurodegeneration of the ganglion cell complex (GCC), correlating with memory impairments.

SAC and CBD exhibited significant therapeutic effects against CNS pathophysiologies, attenuating glial activation and synergistically preventing BBB leakage. In the retina, these compounds attenuated BRB leakage risk and reduced glial-mediated neuroinflammation. SAC and CBD preserved GCC thickness and improved memory functions, proving to be as effective as or superior to metformin against diabetic-associated CNS pathophysiology

Abbreviations:

8OHdG, 8-Hydroxyguanosine; BBB, blood brain barrier; BRB, blood retinal barrier; CBD, cannabidiol; CNS, central nervous system; CTX, cortex; GCC, ganglion cell complex; GFAP, glial fibrillary acidic protein; HPF, hippocampal formation; HOMA-IR, Homeostasis model assessment–insulin resistance; IgG, immunoglobulin G; Iba1, ionized calcium-binding protein 1;

INL, inner nuclear layer; ONH, optic nerve head; NVU, neurovascular unit; ONL, outer nuclear layer; OCT, optical coherence tomography; SAC, S-allyl-cysteine; TyG, Triglyceride-glucose; T2D, Type 2 diabetes mellitus

Keywords:
Cannabidiol; Cognitive dysfunction; Glial activation; Neurovascular; S-allyl-cysteine; Type 2 diabetes mellitus

3.2.2 Introduction

Cognitive dysfunction is recognised as a prevalent complication and comorbidity of type 2 diabetes mellitus (T2D) [1] and accumulating evidence supports the role of cerebrovascular dysfunction, chronic neurovascular inflammation and oxidative stress as causal risk factors [2,3]. Recent studies on the retina suggest a microvascular axis for cognitive decline in T2D, with a significant association between retinal capillary integrity, retinal neurodegeneration and deficits in memory functions [4–6]. Clinical neuroimaging studies have linked T2D-associated cognitive dysfunction to BBB disruptions and atrophy in cognition-related brain regions [7,8]. Animal studies support these findings, demonstrating that neurovascular breakdown and glial-mediated neuroinflammation contribute to learning and memory deficits [9,10]. Our previous studies on diet-induced insulin resistance have indicated that vascular pathology precedes cognitive impairment [11], suggesting that therapeutics targeting microangiopathy and glial overactivation may improve cognitive outcomes.

The deterioration of the neurovascular unit (NVU) observed in T2D involves the impairment of both the blood-brain barrier (BBB) and blood-retinal barrier (BRB) [12]. Emerging data indicate that astrogliosis and chronic activation of resident microglia facilitate the breakdown in NVU functionality [8,9]. The state of reactive gliosis, mediated by diabetic metabolic dysregulation, leads to over-production of reactive oxygen species (ROS) and a concurrent reduction in antioxidant capacity. These factors contribute to cellular oxidative stress, which manifests in DNA damage and ultimately, neuronal cell loss [10,11]. These findings underscore the need for interventions aimed at positively modulating glial cell activation and preserving neurovascular

integrity. Such approaches may mitigate the risk of cognitive decline and ocular degenerative pathology in the context of T2D.

Naturally derived biomolecules boasting anti-inflammatory and antioxidant properties offer a promising, safe and cost-effective approach to the treatment of metabolic [12,13], vascular [14,15] and neurodegenerative [16,17] disorders. Two such compounds, s-allyl-cysteine (SAC) derived from aged garlic (Allium sativum) and cannabidiol (CBD) from the *Cannabis sativa* plant, have garnered attention as neuroprotective agents with therapeutic potential in T2D complications within neural tissues.

SAC, an organosulfur molecule and a potent ROS scavenger, is reported to improve spatial memory recognition and mitigate oxidative stress in senescence-accelerated SAMP8 and SAMP10 mice [22] and in a murine model of type 1 diabetes mellitus (T1D) [23]. Moreover, SAC exhibits neurotrophic effects in inflammatory-induced cognitive dysfunction, characterised by attenuation of astrogliosis and a reduction in microglial immunoreactivity [24]. Clinical studies that administer SAC as a garlic supplement have also shown its potential to slow retinal neurodegeneration and enhance visual acuity in T2D [25]. However, the mechanisms underlying the beneficial effects of SAC in T2D have yet to be directly explored.

CBD, the major non-psychotropic phytocannabinoid, showcases pleiotropic cellular and physiological effects in murine models of T1D, mild traumatic brain injury, and virus-induced demyelination [22–24]. CBD consistently exhibits the capacity to reduce proinflammatory cytokines, attenuate glial cell activation, mitigate BBB hyperpermeability, and inhibit neurodegeneration [29]. Furthermore, exogenous provision of CBD has conferred protection of cognitive function in models of Alzheimer's disease, in part through the modulation of the endocannabinoid system [30]. Consequently, it is imperative to examine the physiological response to exogenous CBD in the context of cognitive performance in T2D.

This study was designed to investigate the mechanisms underlying the supposed protective effects of SAC and CBD in maintaining integrity of the NVU within the brain and retina, with the aim of preventing cognitive dysfunction in a robust model of T2D. Furthermore, this investigation explores the potential synergistic therapeutic effects of combined SAC+CBD treatment, which

remains a novel area of exploration. Using the widely adopted leptin receptor-deficient db/db mouse model of T2D, we hypothesise that SAC and CBD, administered individually or in combination, may ameliorate BBB and BRB hyperpermeability through the modulation of microglial and astrocytic immunoreactivity, thereby mitigating the burden of neuroinflammation and oxidative stress. Provision of SAC and/or CBD may preserve memory function and retinal neuronal structure in T2D.

3.2.3 Results

SAC and CBD attenuate markers of metabolic dysregulation linked to diabetes

This study investigated the potential metabolic effects of SAC and CBD in db/db mice with T2D phenotype (Fig 3.2). Diabetic db/db control mice showed significant hyperglycaemia, hyperinsulinemia and hypertriglyceridemia (Fig 3.2A-C). Surrogate markers of insulin sensitivity demonstrated resistance in both glucose (HOMA-IR) and lipid (TyG) metabolism contexts (Fig 1D-E). Additionally, a significant increase in body weight was observed in the db/db control mice in comparison to nondiabetic db/+ controls (Fig 3.2F).

Dietary intervention with SAC markedly reduced diabetic insulin and triglyceride levels, maintaining the TyG measure of insulin sensitivity at levels comparable to nondiabetic db/+ controls. There was no significant impact on body weight in SAC treated db/db mice. The positive metabolic effects of SAC were found to be equipotent, or better than those observed db/db mice treated with metformin.

Treatment with CBD in db/db mice led to a paradoxical increase in plasma glucose that reached statistical significance. However, all treatment groups indicated a reversed trend in plasma glucose levels after the 23-week intervention. The therapeutic effects of CBD on metabolic status were primarily mediated by insulin, with a significant reduction in plasma insulin levels and suggestive improvements in HOMA-IR insulin resistance, although not the TyG score per se. The CBD-associated reduction in plasma triglyceride concentration was non-significant and comparatively less in magnitude than that of other treatment groups. Notably, CBD monotherapy showed the only significant attenuation of obesity in db/db mice.

Surprisingly, treatment with SAC+CBD combination in db/db mice demonstrated no synergistic improvements in the T2D metabolic phenotype compared to monotherapy interventions. SAC+CBD treatment resulted in comparable levels of plasma glucose to the single therapy groups, but no additional benefits were observed in lowering insulin levels. Administration of SAC+CBD normalized plasma triglycerides with similar efficacy to SAC treatment alone. However, no significant improvements were observed in markers of insulin sensitivity, as determined by HOMA-IR or TyG indices, in SAC+CBD-treated mice. Notably, there was no evidence of enhanced efficacy in insulin sensitivity for the SAC+CBD group compared to the single therapy groups. Treatment with the SAC+CBD combination also showed no therapeutic benefits for body weight in obese db/db mice.

The oral hypoglycaemic metformin showed weak therapeutic effects against the diabetic metabolic phenotypes. Dietary metformin for 23 weeks did not positively regulate glucose homeostasis, but rather significantly exacerbated plasma glucose concertation in db/db mice. Treatment with metformin significantly reduced plasma insulin and triglyceride levels; however, it did not confer positive effects in their respective measures of insulin sensitivity, HOMA-IR and TyG index. Body weight also remained elevated in metformin-treated db/db mice compared to nondiabetic db/+ controls.

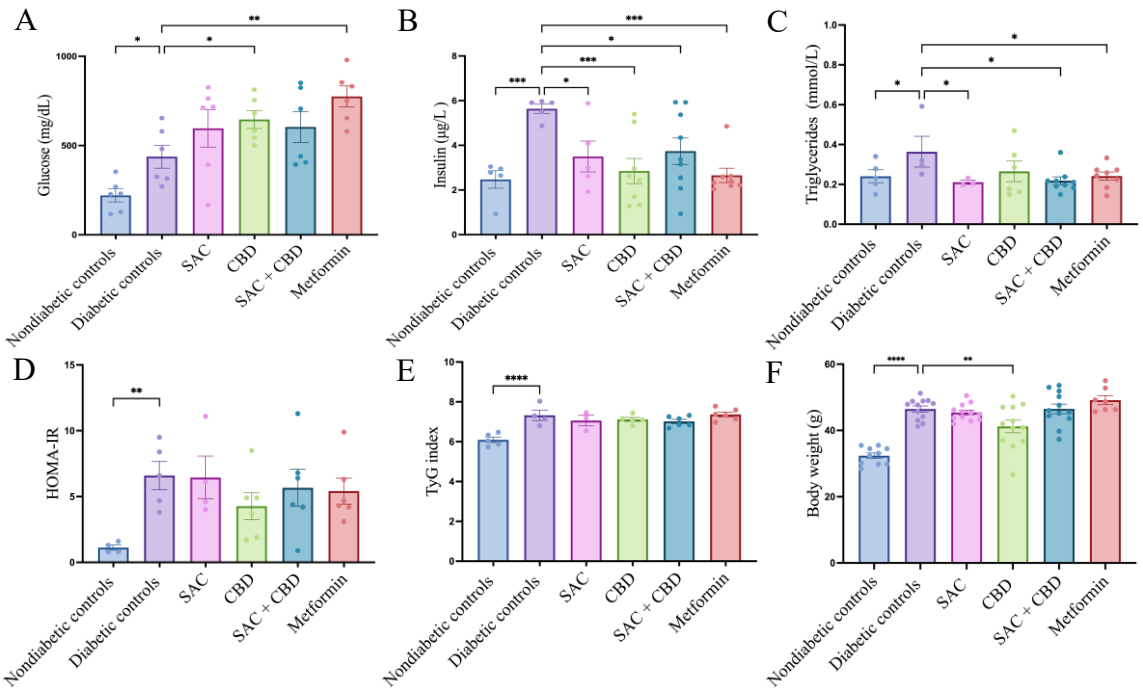

Figure 3-2 Metabolic parameters after 23 weeks of dietary intervention.
Measures indicated are for plasma glucose (A), insulin (B), and triglyceride levels (C), HOMA-IR (D) and TyG index (E) as calculations of insulin resistance, and body weight (F). The intervention groups were 1) nondiabetic db/+ controls, 2) untreated diabetic db/db controls, 3) db/db mice maintained on SAC (50mg/kg), 4) CBD (75mg/kg), 5) SAC+CBD (50mg/kg and 75mg/kg, respectively), or 6) metformin (200mg/kg). The data is shown as mean +/- SEM. Statistical significance was assessed at ** *p<0.01 and* **** *p<0.0001* using ANOVA with Fisher's LSD post-hoc test. Animals used: n= 8-10.

SAC and CBD mitigate diabetic neuroinflammation and oxidative stress, and synergistically protect BBB integrity

The ameliorative potential of SAC and CBD against BBB leakage, glial activation and oxidative damage were evaluated through immunofluorescence neuroimaging of the hippocampal formation (HPF) and cortex (CTX) (Fig 3.3).

BBB permeability, assessed by the immunoreactivity of extravasated IgG within the brain parenchyma (Figs 3.3A-C), was over threefold greater in db/db controls compared to nondiabetic db/+ controls. The significant increase in BBB leakage was noted in both the CTX and HPF regions (Fig 3.3D). Treatment with SAC and CBD monotherapy suggested some improvement in BBB integrity in the HPF, with an average reduction in parenchymal IgG by 50%, approaching statistical significance. In contrast to metabolic measures, SAC+CBD combination therapy demonstrated synergistic BBB protection against IgG extravasation. Significant reductions in IgG abundance were evident in both brain regions, with a more pronounced effect in the HPF. Metformin treatment also significantly reduced IgG abundance in the brain.

Astrogliosis was determined as GFAP immunoreactivity (Fig 3.3E). Diabetic db/db controls showed a significant increase in GFAP–astrocyte reactivity primarily in the hippocampus, consistent with the observed BBB breakdown. Dietary SAC and particularly CBD essentially abolished astrocyte reactivity in db/db mice, with GFAP abundance markedly decreased even compared to nondiabetic db/+ controls. Metformin significantly decreased GFAP immunoreactivity in db/db mice, but the observed improvement was modest compared to naturally

derived agents, with significant differences in efficacy observed compared to CBD-containing formulations.

The pathological increase in BBB leakage and astrogliosis was also mirrored by the increase in Iba1-microglial activation, as the principal measure of neuroinflammation (Fig 3.3F). The elevated Iba1 immunointensity in db/db mice was normalised by SAC and CBD monotherapies and combination treatment. Metformin also significantly suppressed Iba1-microglial immunointensity.

Oxidative damage to mitochondrial and nuclear DNA, an established pathophysiology of T2D, was evaluated through 8OHdG immunointensity (Fig 3.3G) and showed minimal increase in db/db mice compared to nondiabetic db/+ mice. However, CBD- containing formulations nevertheless displayed substantial reductions in 8OHdG staining. In contrast, the SAC and metformin interventions showed no apparent effect on DNA oxidation. Representative images of glial activation and DNA oxidation are shown in Figure 2H-J.

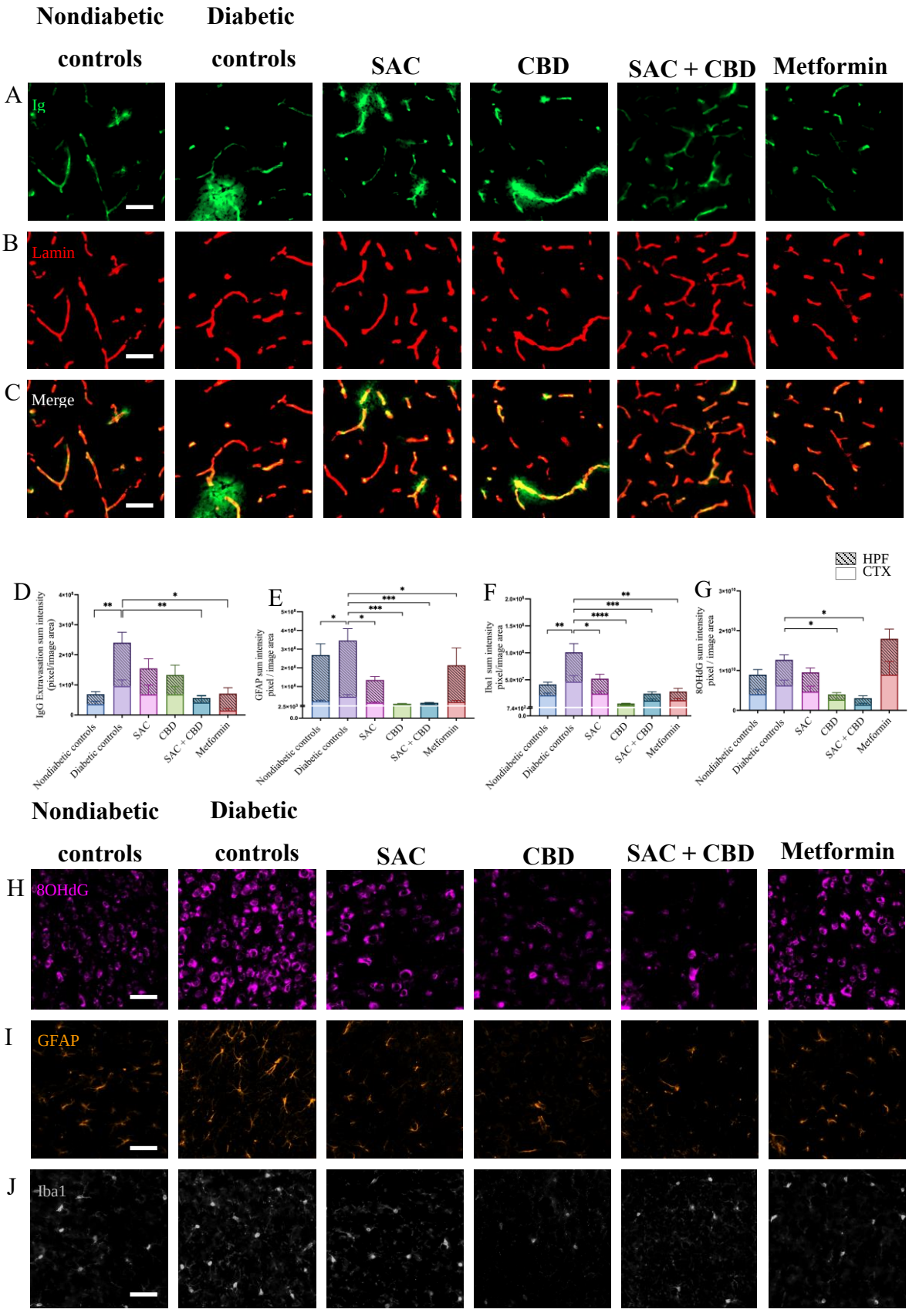
Nondiabetic controls
Diabetic controls
SAC
CBD
SAC + CBD
Metformin
A
Ig
B
Lamin
C
Merge
D
IgG Extravasation sum intensity (pixel/image area)
E
GFAP sum intensity pixel / image area
F
Iba1 sum intensity pixel/image area
G
8OHdG sum intensity pixel / image area
HPF
CTX
Nondiabetic controls
Diabetic controls
SAC
CBD
SAC + CBD
Metformin
H
8OHdG
I
GFAP
J
Iba1

Figure 3-3 Brain immunofluorescence measures.
Representative images for each experimental group of co-immunostaining for IgG in green (A) and Laminin in red (B), which are merged to identify BBB leakage (C). The immunointensities for extravasated IgG that signals BBB leakage (D), as well as GFAP as a marker of astrogliosis (E), Iba1 as an indicator for microglia activation (F), and 8-OHdG that indicates DNA oxidation (G) are presented following a 23-week treatment intervention. The cortex (bottom) and hippocampal formation (top) are shown in the stacked graphs. Corresponding representative images for GFAP in orange (H), Iba1 in grey (I), and 8OHdG in pink (J) are also provided. White scale bars equal 50 μm. Immunofluorescence measures were assessed in nondiabetic db/+ controls and diabetic db/db mice, and across all treatment groups (SAC 50mg/kg, CBD 75mg/kg, SAC+CBD 50mg/kg and 75mg/kg, respectively and metformin 200mg/kg). Statistical significance with Fisher's LSD is noted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$, ****$P<0.0001$. Animals used: n= 5-12.

Natural compounds prevented diabetes-associated thinning in the inner retina

Retinal OCT imaging was utilised for the *in vivo* assessment of thickness of the total neuroretina and relevant sublayers at the optic nerve head, as indicated in the representative images (Figs 3A-C). Although the thickness of the total neuroretina was comparable between diabetic db/db and nondiabetic db/+ controls, there was a significant increase in thickness following a 23-week SAC+CBD and metformin intervention (Fig 3.4D).

The diabetes-associated thinning of the GCC was evident. However, db/db mice treated with SAC and CBD, alone and in combination, exhibited GCC preservation with equal effectiveness as metformin treated mice (Fig 3.4E).

The INL and ONL, as dense cellular and synaptic regions of the retina, were examined for diabetic thinning. The INL did not show significant differences in db/db mice compared to nondiabetic controls. However, among the interventions tested, the SAC+CBD combination displayed a marked increase in INL thickness (Fig 3.4F). In contrast, the ONL was significantly thicker in db/db controls relative to nondiabetic controls (Fig 3.4G). Notably, db/db mice that received either SAC+CBD combination or metformin indicated significant increases in ONL thickness.

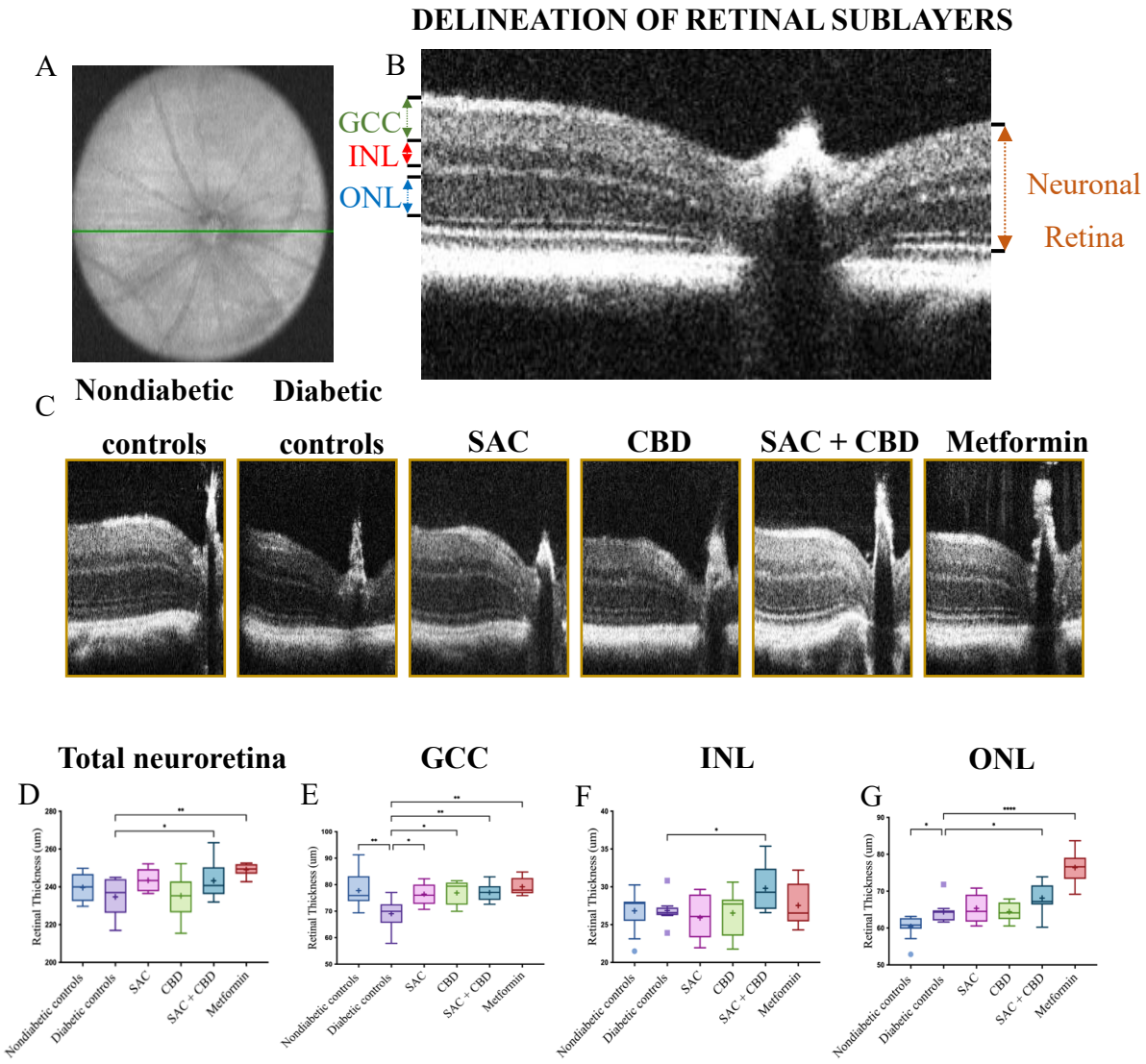

Figure 3-4 Retinal OCT imaging measures.

Representative OCT image of the central en-face scan with the optic nerve head shown by a green line (A), and corresponding B-scan of the central retina of a nondiabetic control with relevant retinal layers indicated (B). Representative images for each experimental group are provided to demonstrate differences in thicknesses (C), with white scale bar representing 50 μm. Retina thickness for total neuroretina (D), GCC (E), INL (F), ONL (G), and shown as box and whisker plots, with a line at the median, and a '+' symbol at the mean after 23 weeks of treatment. Experimental groups: nondiabetic db/+ controls, diabetic db/db controls, SAC (50mg/kg), CBD (75mg/kg), SAC+CBD (50mg/kg and 75mg/kg, respectively), and metformin (200mg/kg). Statistical significance using Fisher's LSD is noted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$,

*****P<0.0001*. GCC, ganglion cell complex; INL, inner nuclear layer; ONL, outer nuclear layer. Animals used: n= 6-12.

Diabetic astrogliosis and neuroinflammation in the retina were prevented with natural compounds and metformin.

Following *in vivo* OCT imaging, retinal wholemounts were assessed for neurovascular and glial pathophysiology (Figs 3.5A-C). The data confirm parallel mechanisms of neurodegenerative thinning as observed in diabetic cerebral pathology.

Consistent with the assessment of BBB leakage, retinal extravasation of IgG was detected in 8% of db/db mice, while no such no leakage was observed in nondiabetic db/+ control mice (Fig 3.5D). Approximately 40% of BRB leakage events occurred in db/db controls. Among the dietary intervention groups, SAC-treated mice accounted for 40% of the BRB leakage events; however, there was a tenfold decrease in the immunointensity of extravascular IgG in the retina parenchyma. SAC+CBD-treated mice represented 20% of BRB leakage events and demonstrated a further tenfold decrease in IgG immunostaining. Treatment with CBD monotherapy or metformin showed total protection of BRB with no observable IgG extravasation.

In alignment with the BRB breach, corresponding changes in GFAP-astrogliosis and Iba1-microglial neuroinflammation were observed in the db/db retina (Fig 3.5E). Astrogliosis was increased in db/db mice, and all treatment interventions showed significant reductions in GFAP immunointensity. Although no statistical differences were observed among the intervention groups, CBD-containing therapies suggested greater efficacy in attenuating astrogliosis.

The abundance of Iba1 immunointensity more than doubled in db/db control mice compared to nondiabetic controls (Fig 3.5F). However, the provision of SAC and CBD monotherapy and combination treatments completely prevented the increase in Iba1-microglial activation in db/db retinas. Notably, dietary intervention with natural compounds demonstrated equipotent efficacy to metformin in suppressing microglia Iba1 abundance in the retina.

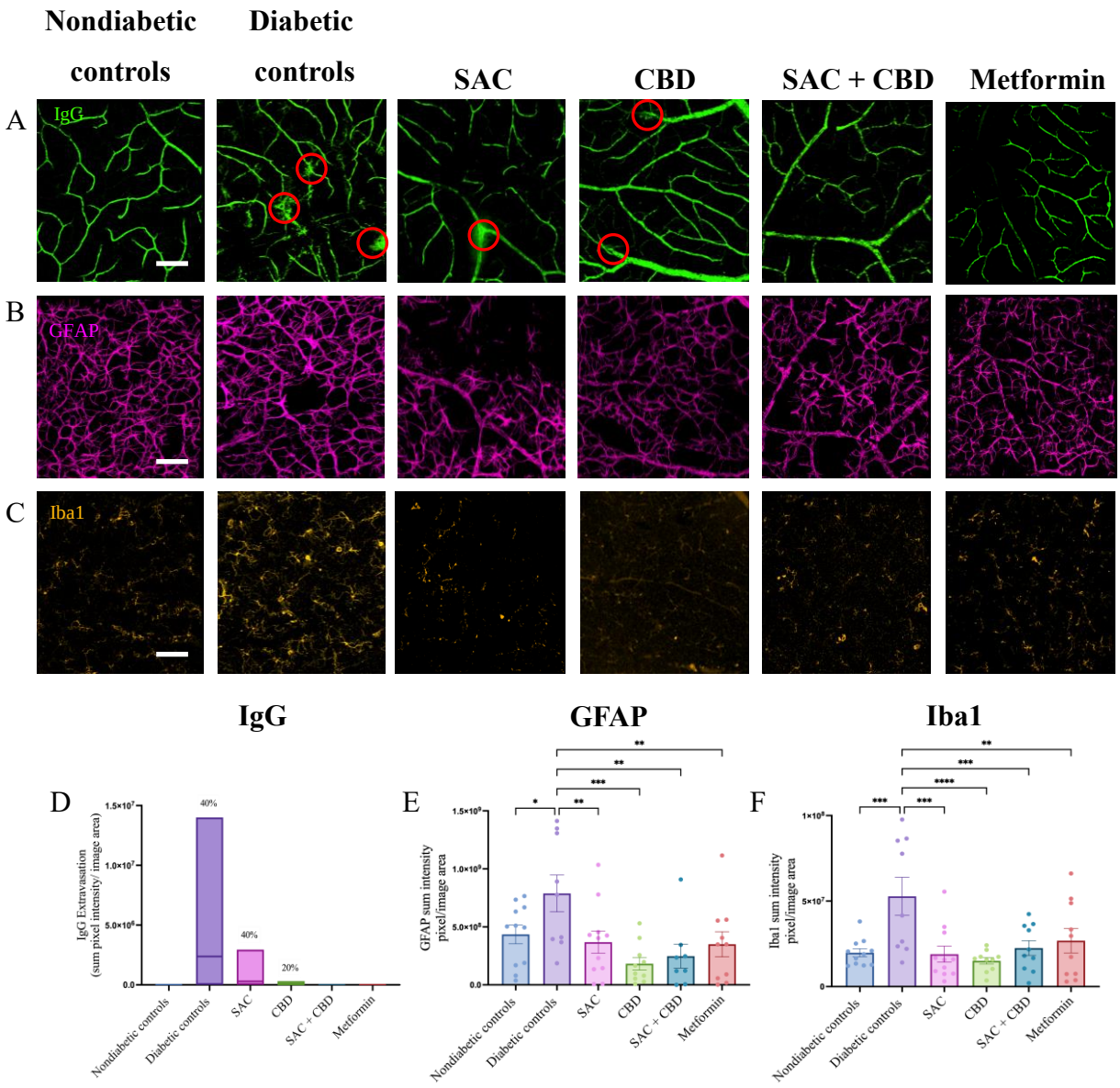

Figure 3-5 Retinal immunofluorescence images per experimental group.

IgG is represented in green (A), with examples of leakage indicated by the red circle. Immunostaining for GFAP in pink (B) and Iba1 in orange (C) shows variable abundance across experimental interventions. White scale bars represent 50 μm. Immunointensity of extravascular IgG (D), GFAP-labelled astrocytes (E), and Iba1-labelled microglia (F) in retinal wholemounts after a 23-week treatment intervention. Experimental groups were nondiabetic db/+ controls, diabetic db/db controls, SAC (50mg/kg), CBD (75mg/kg), SAC+CBD (50mg/kg and 75mg/kg, respectively), and metformin (200mg/kg). Statistical significance for GFAP and GFAP measures

using Fisher's LSD is noted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.001$. Animals used: n= 8-10.

SAC and CBD improve memory impairment with greater efficacy than metformin

To determine whether the observed improvements in neurovascular integrity and glial cell activation conferred benefits in cognitive function, we conducted two functional tests of memory (Fig 3.6).

In the NOR test for short-term memory, lower preference index scores reflect a failure to identify and explore the novel object. The NOR test revealed significant decrease in preference index in db/db mice compared to nondiabetic db/+ controls (Fig 3.6A). In line with the beneficial effects of SAC and CBD on the neuroinflammatory milieu, 23 weeks of treatment resulted in complete preservation of short-term memory. While SAC+CBD prevented the decrease in the preference index, no further additive effects were realised with combination therapy. Interestingly, metformin was less effective in supporting performance in the NOR test compared to other treatment groups, especially SAC or CBD monotherapy.

The PA test for long-term memory showed a diabetes-associated impairment consistent with findings from the NOR challenge (Fig 3.6B). There was a significant decrease in the latency time recorded for db/db mice compared to db/+ controls, indicating an inadequate fear memory response. After the 23-week intervention, SAC conferred significant improvement in latency, while CBD presented non-significant improvements compared to untreated diabetic db/db control mice. Treatment with SAC+CBD combination showed a lesser therapeutic effect than the single therapy groups. Similarly, metformin displayed minimal efficacy on PA latency, resembling the SAC+CBD combination group.

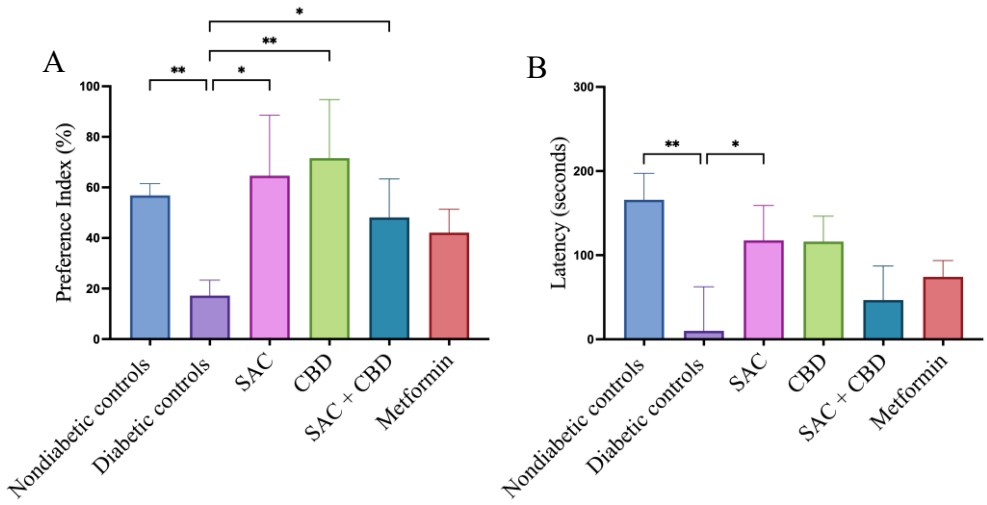

Figure 3-6 Cognitive outcomes in short-and long-term memory tests.

Preference index during the test phase of the novel object recognition (NOR) test as a measure of short-term memory performance after 23 weeks of treatment intervention (A). The passive avoidance test of long-term memory is indicated as latency in seconds (B). Memory function was assessed across all experimental groups: nondiabetic db/+ controls, diabetic db/db controls, and diabetic mice on treatment interventions: SAC (50mg/kg), CBD (75mg/kg), SAC+CBD (50mg/kg and 75mg/kg, respectively), and metformin (200mg/kg). Statistical significance using Fisher's LSD is noted as follows: *$P<0.05$, **$P<0.01$, ***$P<0.00$, ****$P<0.0001$. Animals used: n= 2-12.

Diabetic metabolic status is associated with pathological changes in the brain and retina

SAC and CBD ameliorated CNS pathology such as glial immunoreactivity yet showed minimal significant outcomes in metabolic parameters. Examination of the connection between plasma metabolic phenotypes and markers of diabetic CNS pathology in untreated animals is relevant for interpretation of these results. Plasma data aggregated from the nondiabetic db/+ and diabetic db/db control mice at 28 weeks alongside matched control animals at 14 weeks of age. Importantly, there were no significant differences in the outcomes of the nondiabetic db/+ controls at 14 and 28 weeks of age. This suggests that age-related effects did not influence the associations observed in our correlation analyses.

Pearson's correlation coefficient analysis (Fig 3.7) revealed significant associations between metabolic status and CNS pathology in diabetes. There was a significant association between elevated plasma glucose and Iba1- microglial activation in the brain. The most consistent finding was a moderate to strong association between poor insulin regulation and NVU injury, reflected in significant correlation coefficients for BBB leakage, GFAP-astrogliosis and Iba1-microglial activation. The HOMA-IR measure of insulin resistance was also significantly associated with retinal astrogliosis. There were no significant associations between plasma metabolic parameters and cerebral oxidative stress marker, 8OHdG or retinal Iba1- microglia staining, however the data indicated trends of weak to moderate positive and negative correlations, respectively. Furthermore, plasma triglycerides showed no significant associations with markers of neuroglial and vascular injury in the CNS.

There were reversed correlations between metabolic parameters and memory performance; trends deviated from insulin-centric associations and varied according to the memory test employed. Preference index in the NOR test of short-term memory was positively correlated with disrupted metabolic status; most notably with plasma glucose and insulin resistance relative to lipid metabolism indicated by TyG index. Conversely, the PA test of long-term memory showed weak to strong negative associations, whereby a decrease in latency was significantly correlated elevated plasma glucose and TyG index.

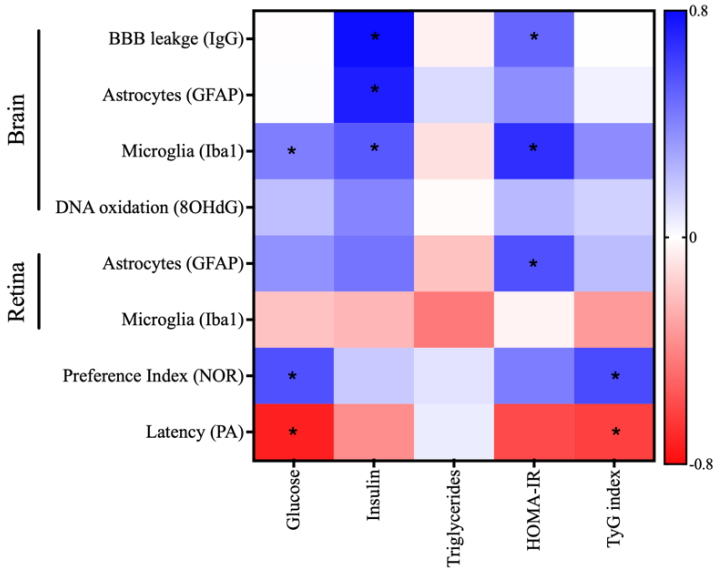

Figure 3-7 Correlation matrix of metabolic status against immunofluorescence markers for CNS pathophysiology.

This includes BBB leakage, astrogliosis, microglial-associated neuroinflammation, oxidative stress, and cognitive performance. This data presents a deviation from the single-timepoint study design of this intervention study. Notably, the heatmap, with corresponding colour scale that is proportional to the r value of the Pearson's correlation coefficient, represents data aggregated from diabetic db/db mice and nondiabetic db/+ controls on standard chow at 14 weeks and 28 weeks of age. The latter experimental group is consistent with control data from this 23-week intervention study. Statistical significance at $p<0.05$ is indicated with an asterisk (*).

BBB leakage, with associated neuroinflammation and oxidative stress, collectively serve as pathological correlates of diabetic memory impairment

Examining the relationship between neurovascular dysfunction and cognitive impairment not only validates glial and vascular pathology as therapeutic targets for cognitive decline but also provides context for exploring the potential mechanisms of SAC and CBD action. Measures of CNS pathology in diabetic db/db mice and nondiabetic db/+ mice on standard chow (controls) at 14 weeks and the 28-week animals utilised in this dietary study were compiled into Pearson's correlation analyses. Importantly, there were no age-associated effects in nondiabetic db/+ controls across the experimental timepoints.

Pearson's correlation coefficient analyses (Fig 3.8) demonstrated strong associations between cerebral pathophysiological markers and memory impairment in the db/db model of T2D. BBB dysfunction was significantly associated with neuroinflammation, astrogliosis, and oxidative stress (Fig 3.8A); memory impairment directly correlates with BBB leakage and glial activation (Fig 3.8B). The correlations for extravasated IgG immunointensity in Fig 3.8A were positive and strong for GFAP-positive astrocytes, moderate for Iba1-positive microglial activation, and weakest for the 8OHdG marker of DNA oxidation.

The follow-up Pearson's correlation analysis in Fig 3.8B indicated weak to strong negative associations between short- and long-term memory function and various immunofluorescent markers of cerebral pathology. Generally, the correlations were comparable across short- and long-

term memory tests. A decreased preference index was moderately associated with cerebral microglial activation and astrogliosis. No correlation was identified between short term memory function and 8OHdG immunoreactivity. However, there was a strong association between short-term memory impairment and IgG extravasation in the brain.

Long-term memory impairment, signified by decreased latency in the PA test, showed significant associations that were strong for BBB leakage, moderate for Iba1-microglial, respectively, and weak for 8OHdG immunointensity. The association between long-term memory impairment and GFAP-astrocyte reactivity was moderate but did not reach significance.

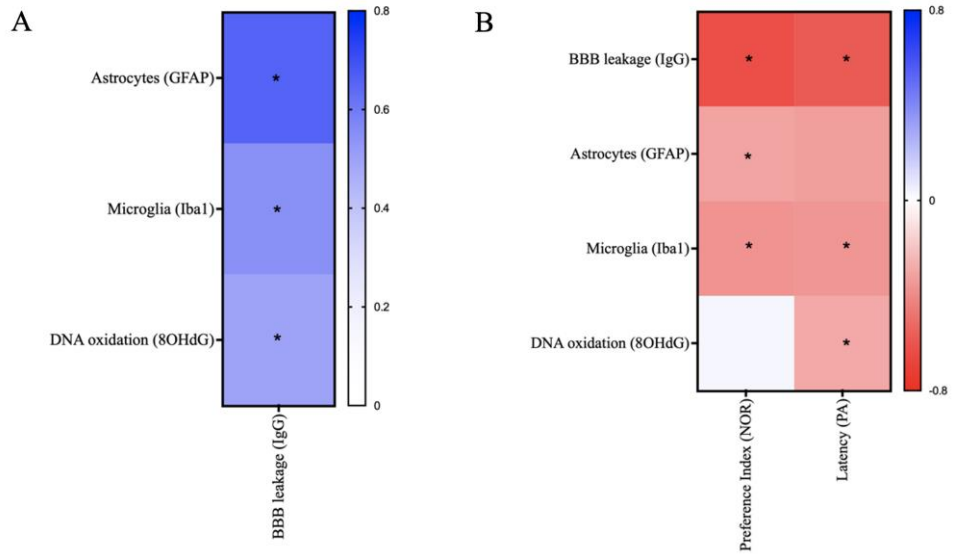

Figure 3-8 Pearson's correlation heatmaps to examine neurological associations of T2D.
The correlation matrix between BBB leakage, as parenchymal IgG extravasation, and diabetic glial pathophysiology (A). A follow-up correlation heatmap shows associations for memory performance tests against brain immunofluorescence markers for neuroinflammation, astrogliosis, oxidative stress and BBB leakage (B). Animals used in the correlation analysis include diabetic db/db mice and nondiabetic db/+ controls on standard chow at 14 weeks and 28 weeks of age. The 28-week animals correspond to this 23-week dietary intervention study. Significance at $p<0.05$ is indicated with an asterisks (*).

3.2.4 Discussion

This study aimed to investigate the individual and combined effects of naturally derived compounds, SAC and CBD, in preserving cognitive function by preventing BBB/BRB disruption, glial cell activation and oxidative DNA damage in the established db/db mouse model of T2D.

Our metabolic measures in db/db mice were consistent with moderate T2D, including hyperglycaemia, hyperinsulinemia, and hypertriglyceridemia, along with insulin resistance and obesity. These moderately diabetic db/db mice displayed impaired short-term episodic memory, as evidenced by a decrease in preference index in the NOR test. Additionally, the mice showed reduced latency time in the PA test, indicating dysfunction in long-term memory formation mediated by the hippocampus and amygdala in response to aversive stimuli [42]. Importantly, our data indicate that dietary intervention with SAC and/or CBD improved short-term memory function, with SAC also preserving long-term memory.

The exact mechanisms by which SAC/CBD prevented cognitive decline in moderately diabetic db/db mice are not fully understood. In the db/db mice, either SAC, CBD, or their combination effectively reduced hyperinsulinemia to a similar extent as the anti-diabetic drug, metformin. These findings align with recent evidence indicating that SAC and CBD enhance insulin receptor sensitivity, lipid metabolism and cellular glucose uptake [43,44]. Notably, SAC demonstrated efficacy in improving hypertriglyceridemia in db/db mice, in line with established lipid-modulating effects of cysteine compounds [16]. Additionally, CBD treatment resulted in a reduction in body weight, supporting recent clinical findings on the potential use of phytocannabinoids in obesity treatment [45]. However, it is crucial to note that the overall benefits of SAC and CBD, whether administered alone or in combination, against metabolic dysregulation were modest, leaving measures of insulin resistance elevated in db/db mice. Similarly, metformin exhibited limited restorative effects on diabetic metabolic status in this study, failing to significantly impact hyperglycaemia and insulin resistance as demonstrated in other studies [46]. These data suggest that the therapeutic effects of SAC and CBD in the CNS may be potentiated through mechanisms independent of glucose and insulin regulation.

Instead, the benefits of SAC and CBD on cognitive performance in db/db mice may attribute to its neurovascular protective anti-inflammatory/-oxidative properties. Indeed, the cognitive-preserving actions of SAC and CBD monotherapies have been reported in multiple disease models, involving vascular protection and amelioration of chronic reactive gliosis and oxidative stress in the CNS [47,48].

Furthermore, the db/db mice exhibited significant cerebral abnormalities such as BBB leakage, reactive gliosis, and oxidative DNA damage. Retinal changes we observed in db/db mice included reduced GCC thickness, increased BRB leakage risk, and significant glial activation, all associated with impaired memory functions. These findings align with existing evidence linking cerebral and retinal microangiopathy, neuroinflammation, and oxidative stress to cognitive dysfunction and neural degeneration in the context of T2D [6,12]. Interestingly, Corem et al., [49] reported transient BBB disruption in normoglycemic db/db mice, which disappeared upon diabetes onset. Emerging evidence suggests that both transient and prolonged BBB disruptions involve astrocyte reactivity and microglial activation in T2D and other neurodegenerative disorders [50]. Additionally, activated glia contribute to oxidative stress, leading to DNA mutations and cellular damage that exacerbates neuroinflammation [51].

Remarkably, treatment with natural agents conferred substantial neuroprotective effects in diabetic db/db mice. SAC and CBD, either alone or in combination, demonstrated potent anti-inflammatory effects, reducing GFAP-astrogliosis and Iba1-microglial activation. The SAC+CBD combination treatment notably restored BBB integrity more effectively than monotherapies. These findings are supported by previous studies, with SAC shown to decrease oxidative DNA damage in cerebral ischemia [52] and ameliorate reactive gliosis in the streptozotocin (STZ) rat model of diabetes [23]. CBD has been reported to attenuate BBB leakage and microglial activation in a viral multiple sclerosis model [27] and modulate astrocyte activity through the endocannabinoid system, suggesting potential therapeutic benefits for various neurological disorders [53].

Due to physiological similarities, we recently reported that the microvascular dysfunction and neuroinflammatory cascades in the diabetes brain are closely mirrored in the retina, leading to thinning of GCC cell layer [6]. Immunofluorescence analysis in this study showed an increased

risk of focal BRB leakage, accompanied by GFAP-astrogliosis and Iba1-microglial activation in diabetic db/db mice. The hippocampus's vulnerability to glial pathology exceeded that of the cortex, as previously reported [6]. Evidence from T2D models suggests that BRB leakage and glial-mediated neuroinflammation contribute to retinal ganglion cell death [13,54]. Importantly, our study provided novel evidence that SAC and CBD, whether alone or combined, provide neuroprotection against GCC thinning comparable to metformin. This preservation of retinal thickness may be due to reduced BRB leakage risk and severity, as well as complete protection against abnormal glial activation in the retina.

Although research on SAC and CBD in ocular diseases is limited, SAC has demonstrated potential to suppress GFAP-astrocyte immunoexpression in kainate-induced toxicity [55]. Similarly, CBD has been reported to reduce the expression of inflammatory cytokines and pro-adhesion molecules, which promote vascular permeability and cell death [28]. These reports suggest that SAC and CBD promote retinal health through modulation of glial cell activation.

Mechanistic research supports the effects of SAC and CBD on neurocognition. SAC's antioxidant actions involving nuclear factor (erythroid-derived 2)-like 2 (Nrf2) have been shown to preserve spatial learning and memory functions, along with immunomodulatory effects that down-regulate pro-inflammatory nuclear factor kappa B (NF-κB) cascades and reduce gliosis in the hippocampus [24], [56]. Similarly, CBD demonstrated benefits in improving episodic memory and social learning in models of Alzheimer’s disease, attributed to its modulation of various pathways involved neuroprotection, including the NF-κB/Nrf2 pathway [57]. CBD is also shown to activate nuclear receptors such as peroxisome proliferator-activated receptor gamma (PPARγ), which downregulates the expression of inflammatory genes in glia cells [58]. Overall, the neuroprotective effects of SAC and CBD observed in this study are supported by their anti-inflammatory and antioxidative actions, which may help mitigate memory impairment in T2D.

In contrast to SAC/CBD, metformin showed limited protection against glial-mediated neuroinflammation, despite a protective effect on the BBB. The modest improvements in memory function observed in metformin-treated db/db mice in this study may be due to its limited efficacy in neuroprotection. Contrary to our findings, De Oliveira et al., [59] reported substantial

therapeutic benefits of metformin in STZ-induced T1D mice, including mitigation of spatial memory decline, astrocyte and microglial activation, and reduced levels of pro-leakage vascular endothelial growth factor (VEGF). Likewise, Pilipenko et al., [60] documented improved cognitive function and reduced reactive gliosis in toxin-induced Alzheimer's disease models. However, recent findings suggest minimal cognitive benefits with metformin treatment [61], and earlier studies even indicate that metformin may exacerbate neuroinflammation [62]. These discrepancies warrant further investigation and suggest that while metformin is an effective anti-diabetic medication, it may not be the optimal therapeutic choice for addressing CNS complications.

Clinical evidence highlights the impact of metabolic dysregulation on neurovascular injury and cognitive deficits [63]. Our study re-affirmed the association between CNS pathology and the metabolic profile of T2D, particularly disruptions in insulin regulation. The central hypothesis of our study proposed that SAC and CBD could preserve cognitive function, in part, by ameliorating the underlying cerebral pathophysiology. To verify this hypothesis, we investigated the relationship between BBB leakage and makers of glial activation and DNA oxidation. Additionally, we investigated the links between memory performance and cerebral pathophysiologies in experimental T2D.

Our study revealed significant correlations between BBB leakage and GFAP-astrogliosis, as well as Iba1-microglial activation, which are consistent with existing literature [64]. Under diabetic conditions, reactive astrocytes exhibit reduced functionality [65], leading to retraction of astrocytic end-feet from microvessels [66]. Similarly, pathologically active microglia promote vascular permeability by producing inflammatory cytokines and engaging in abnormal phagocytosis of neurovascular elements [67]. Our correlation findings emphasise the role of reactive gliosis in the pathogenesis of BBB leakage in T2D. Unsurprisingly, impairments in short-term and long-term hippocampal memory functions were associated with BBB leakage, as well as glial activation. Therefore, the cognitive benefits of SAC and CBD formulations may be linked to their actions on neurovascular and glial components.

While our study provides valuable insights, certain aspects warrant further investigation. For instance, we observed significant changes in the total neuroretina and nuclear sub-layers (INL and

ONL) following treatment with SAC and/or CBD and metformin, despite the absence of apparent diabetes-related changes. Research on the diabetic changes in INL and ONL thicknesses is inconclusive, and these sub-layers may not be prime targets of diabetic neurodegeneration [68], [69]. Additionally, our *ex vivo* assessments of the retina revealed a lower incidence of BRB leakage compared to previous studies in the same model [70]. These disparities may be explained by variations in immunofluorescence markers of BRB leakage and the significant differences in experimental timelines.

It is important to acknowledge the limitations of our study. The use of a single experimental time-point precludes the temporal characterisation of CNS injury in T2D. Another important limitation of our research design is consideration of gender mediated effects. Clinical evidence suggests that women with may experience a higher risk of vascular diseases and cognitive impairments than men [71]. Consequently, future studies should include both sexes to provide more globally representative insights into T2D. While male db/db mice have been shown to exhibit greater cognitive impairments and vascular pathologies compared to their age-matched female counterparts [72], further investigations are needed to examine sex-specific differences within the CNS and assess the effects of SAC and CBD across sexes.

The diabetic db/db model is linked to a hypo-locomotive phenotype [73]. Our study provides new insight that the established NOR protocol may require optimisation for db/db mice, given the substantial exclusion rates in the Familiarisation phase of the test especially in the SAC and CBD monotherapy groups. These results should be interpreted cautiously. Conversely, a study by [74] suggested that db/db mice were suitable for assessments using the open field paradigm, including the NOR test. This discrepancy presents an exciting opportunity for future studies to optimise a broader panel of cognitive tests suitable for the db/db obese phenotype.

Regarding *ex vivo* measures, the assessment of neuroinflammation could have been strengthened by additional examination of proinflammatory cytokines and/or other secretary products of microglia and reactive astrocytes. The inclusion of immunofluorescence markers such as vimentin and S100B for astrocytes, as well as CD65 for microglia, would provide more comprehensive evidence of glial activation. Similarly, the retina could be assessed for additional measures of

neurodegeneration, such as expression of TUNEL or apoptotic-positive cells. The investigation of glial pathology using cross-sectional retinal scans, in contrast to wholemount preparations, may offer a more precise evaluation of oedema and inflammation *via* GFAP-positive Müller cell activation [75]. Given their positioning across most layers of the retina and the abundance of astrocytes around the superior vascular plexus, it is almost impossible to meaningfully delineate Müller cell phenotypes in retinal wholemounts.

The phenotype of experimental and clinical T2D includes hypertension, systemic inflammation, and peripheral vascular abnormalities. While these measures extend beyond the scope of our study, they require investigation to elucidate the broader effects of SAC and CBD in therapeutic management of T2D.

In conclusion, our study enhances the understanding of T2D-related complications in the CNS. The findings demonstrate that SAC and CBD are neuroprotective, with the capacity to prevent neurovascular lesions, glial activation, neurodegeneration, and oxidative injury. These effects contribute to preserving memory function and support the use of naturally derived agents in diabetes therapy.

3.2.5 Materials and methods

Ethics statement

The experimental procedures conducted in this study were approved by the Curtin Animal Ethics Committee (approval no. ARE 2018-19). This research was carried out in full compliance with the Australian Code for the Care and Use of Animals for Scientific Purposes (8th Edition, 2021 updated version), and in adherence to Curtin University's strict ethical standards and the ARRIVE guidelines for reporting experiments involving animals. Efforts were made to minimise animal suffering and to reduce the number of animals used, consistent with ensuring adequate statistical power. Our data analysis procedures strictly followed ethical standards, and the findings are reported with full transparency, including a clear acknowledgment of any limitations.

Animals and treatment intervention

Male C57BLK/6J mice bearing spontaneous monogenetic mutations in the leptin receptor (*db/db*), along with their heterozygote *db/+* counterparts, were acquired from the Jackson Laboratory (California, USA) and maintained at the Animal Resource Centre (Perth, Western Australia). At 4 weeks, mice were transferred to the Animal Facility at Curtin University (Perth, Western Australia. Following 7 d of acclimatisation, animals were randomised to dietary intervention group for 23 weeks, which corresponded to a 28-week experimental endpoint, respectively. The groups were: 1) nondiabetic db/+ controls, 2) diabetic db/db controls, 3) SAC, 4) CBD, 5) SAC+CBD, and 6) metformin. Treatments were incorporated into AIN93M-base chow and thoroughly mixed to homogeneity. To ensure stability, diets were prepared under low-light conditions, cold-pressed into pellets, and packed in 1.5 kg air-sealed bags (Specialty Feeds; Perth, Western Australia). Stock diets were stored in the dark at 4 °C.

Nondiabetic db/+ controls and diabetic db/db controls were fed standard chow. Additional diabetic and nondiabetic controls were maintained on standard chow until a 14-week experimental timepoint, and these animals were used for correlation analyses. For the treatment groups, diets were formulated based on previous evidence of food intake (5-6 g per day) and average body weight of 48g for db/db mice for the duration of experiments [27,28]. SAC (50 mg/kg; 0.04% w/w) was purchased in powder form from ChemScene (New Jersey, USA) and CBD (75 mg/kg; 0.06% w/w) was prepared as a >99% extract and then solubilised at a concentration of 14.5% in Miglyol 812N (medium chain triglyceride carrier oil), and generously provided by Zelira Therapeutics, (Perth, Western Australia). A combination of SAC+CBD (0.04% and 0.06% w/w, respectively) was prepared in the same manner as monotherapy groups, and metformin hydrochloride (200 mg/kg; 0.16% w/w) was obtained in tablet form with vet-approval (Perth, Western Australia). The dosages of these biomolecules and metformin have been previously published with demonstrated therapeutic effects [20, 29–32]. Diets were measured and replenished weekly, and animals had ad libitum access to food and water. Body weight was tracked weekly and immediately prior to euthanasia. Animals were housed in groups within a temperature-controlled laboratory on a 12 h light/dark cycle.

Cognitive Assessment

Novel Object Recognition

The novel object recognition (NOR) test was performed to assess non-spatial recognition memory via cortical-hippocampal neural circuitry as outlined [37] with slight modifications. The test took place in a dedicated behavioral testing room under dim lighting, and a video tracking system was used for automated analysis via the HVS Image 2014 software (HVS Image, UK). On the first day of the procedure, mice were allowed a 10 min habituation period in an empty experimental arena (dimensions: 45 cm x 45 cm x 40 cm). On the second day, mice were placed into to the arena, which had two identical cubic objects positioned diagonally 10 cm from the arena wall. After a 10-minute familiarisation period, the mice were returned to their home cage. The familiarisation threshold was 10 s of active exploration per object. Failure to meet the cut-off resulted in exclusion from the NOR test. To assess short-term memory, one of the previously encountered objects was randomly replaced with a novel, curved object that was distinctly different and unfamiliar to the mice. Following a 2 h delay, mice were placed back into the arena for 5 min of object exploration referred to as the 'test phase'. Exploration was regarded as sniffing or rearing towards an object. A 70% (v/v) ethanol solution was used following each experiment to eliminate odour cues. The Preference Index (PI) was quantified as the proportion of time spent exploring the novel object to the total exploration time for both objects during the 5 min test period, as shown with the formula: Preference index (PI)= Novel object[s]/ (Novel object[s] + familiar object[s]) x 100. Mice were not tested for 24 h after the NOR test.

Passive Avoidance

The step-through Passive Avoidance test was used to evaluate long-term memory function, using a previously described protocol from our lab [38] with minor adjustments (Ugo Basile, Italy). On the first day, mice were placed in the bright compartment of the apparatus, with a light intensity of 1000 lux. After a delay of 30 s, the door to the dark chamber was opened and upon entry, mice received a 2 s, 0.3 mA shock to establish an aversive memory response. The total experimental duration was 300 s. Post shock, the animals were kept in the dark compartment for an additional 10 s before being transferred back to their home cages. Mice that did not enter the darker chamber within the 300 s experimental window were returned to their home cages and subsequently excluded from the assessment. On the second day, after a 24 h interval, mice were reintroduced to the brightly lit chamber under identical experimental conditions. However, the experiment was extended to 345 s, and mice that retreated into the dark chamber were not given a shock. Latency

was calculated as the it took the mice to enter the dark chamber on the second 'test' day minus the time it took on the first 'training' day.

Optical Coherence Tomography

Retinal imaging was carried out according to a previously published protocol [39] with minor amendments. Mice were anaesthetised using ketamine/xylazine (90 mg/kg ketamine, 12.5 mg/kg xylazine). Eye drops of 1% tropicamide and lignocaine (Akorn, Inc.) applied to induce pupil dilation. Retinal imaging was performed on the Bioptigen optical coherence tomography (OCT) system (Bioptigen, Morrisville, NC USA). Volume scans were captured from the optic nerve head (ONH) to the peripheral area of the retinal at the posterior pole of the eye. Cross-sectional B-scan images were stored for subsequent analyses. Measurements were taken of retinal thickness in the following areas: 1) total neuroretina, 2) ganglion cell complex (inclusive of the nerve fibre layer, retinal ganglion cell layer and inner plexiform layer), and 3) inner and outer nuclear layers. Mean thicknesses around the ONH were averaged from 6 measurement points in nasal-temporal regions using FIJI software (ImageJ, US) [40].

Euthanasia and sample collection

Immediately after retinal OCT imaging, blood was collected from deeply anaesthetised mice via cardiac puncture. Mice were then euthanised by cervical dislocation. The brain was removed from cranium and bisected, with the left hemisphere snap-frozen in liquid nitrogen and stored at -80 °C and the right hemisphere fixed in 4% paraformaldehyde overnight then cryopreserved in 20% sucrose for 3 d and frozen using isopentane/dry ice for storage at -80 °C. The right eye was also removed from the head and fixed and cryopreserved as indicated for the above, followed by storage in 0.1% sodium azide at 4 °C. Brains were embedded in Tissue-Tek Optimal Cutting Temperature compound and coronal sections at 20 μm with cortex and hippocampal formation evident were prepared onto Poly-Lysine coated slides (Tranjan) using the Leica CM1520 Cryostat.

Plasma measures for glucose, triglycerides, and insulin resistance

Blood samples were collected in EDTA-coated syringes and subsequently transferred to 1.5 ml Eppendorf tubes for centrifuge at 4 °C for 10 min at 4000 rpm. The resultant plasma supernatant was aliquoted and preserved at −80 °C for metabolic analyses. Non-fasting plasma glucose levels were determined at an optimised dilution ratio of 1:100 using a commercially available

colorimetric assay kit (Abcam). In addition, insulin levels were evaluated using the Ultrasensitive Mouse Insulin ELISA kit (Mercodia), while plasma triglycerides were quantified using a colorimetric assay (Randox). All plasma measures were analysed in accordance with the manufacturers' instructions.

Insulin resistance was calculated using Homeostasis model assessment–insulin resistance (HOMA-IR) with the HOMA Calculator version 2.2.3 (Diabetes Trials Unit), and Triglyceride-glucose (TyG) index according to formula described below:

$$\ln [\text{triglycerides (mg/dL)} \times \text{glucose (mg/dL)}/2]$$

Brain immunofluorescence

The BBB integrity was assessed using a validated protocol for measuring extravasated immunoglobulin G (IgG) in brain parenchyma [41]. Brain sections were co-stained with laminin α4 to indicate the capillary basement membrane and enhance microvascular boundary delineation. To summarise, left hemisphere 20 µm coronal sections were fixed with 4% paraformaldehyde for 10 min at 20 °C. To prevent non-specific binding, sections were blocked using 10% donkey serum (ThermoFisher) for 30 min at 20 °C. The sections were washed in 0.1 M phosphate buffer saline (PBS) and incubated with goat anti-laminin α4 (1:200, RnD Systems) in an antibody signal enhancer solution overnight at 4 °C. Sections were washed in PBS and incubated with donkey anti-goat IgG AlexaFluor 555 followed by goat anti-mouse IgG AlexaFluor 647 mixed in PBS, with each secondary incubation lasting 2 h at 20 °C.

Neuroinflammation represented as reactive gliosis and oxidative DNA damage were evaluated as previously reported [38]. In brief, the right hemisphere 20 µm fixed cryosections of the right hemisphere were rehydrated in PBS, and non-specific binding sites were blocked in 10% donkey serum (ThermoFisher) for 30 min at 20 °C. Sections were incubated overnight at 4 °C with either rabbit anti-ionized calcium-binding protein 1 (1:200, Iba1; Novachem) for microglia or a combination of goat anti-glial fibrillary acidic protein (1:500, GFAP; Abcam) for astrocytes and mouse anti-8-Hydroxyguanosine (1:500, 8-OHdG (15A3); Abcam) for DNA oxidation. Thereafter, sections were incubated with either goat anti-rabbit IgG AlexaFluor 488 (1:500, ThermoFisher) or a combination of donkey anti-goat IgG AlexaFluor 555 (1:1000, ThermoFisher)

and 1:500 donkey anti-Mouse IgG AlexaFluor 647 (ThermoFisher) for 2 h at 20 °C. DAPI was used as a nuclei counterstain.

The cortex and hippocampal formation were captured at 20x magnification (imaging specifications include a OrcaFlash 4.0 camera; Colibri 7 LED-modules 385, 475, 555 and 630nm with penta-band filter set 112HE) on the Zeiss Axioscan Z1 slide scanner (Carl Zeiss, Germany). Images were processed offline using the deep learning features on Zeiss Zen ZEN Blue 3.3 Desk software. To quantitate IgG leakage, the Intellesis trainable segmentation module with machine learning automation capabilities was used based on a protocol developed in-house [41]. Semi-quantitative analysis of protein immunoexpression was determined based on voxel intensity per volume for each region of interest.

Retina immunofluorescence

Retina immunofluorescence staining and imaging were carried out according to an optimised protocol developed in-house. Briefly, retinas were removed from the eye cup and transferred to 96 well-plates containing PBS. They were incubated with Tris-EDTA Buffer (10mM, pH 6.0), overnight at 37 °C, then blocked and permeabilised in a buffer solution made up of 0.2% Tween20, 2% Triton X-100, 0.2% bovine serum albumin in PBS for 1 h at 20 °C with gentle shaking. Retinas were washed in PBS and co-stained for microglia (1:500, Iba1; Novachem) and astrocytes (1:500, GFAP; Abcam) (1:500), overnight at 4 °C with gentle shaking. On the second day of the protocol, retinas were incubated with donkey anti-goat IgG AlexaFluor 647 (1:1000, ThermoFisher) for 2 h at 20 °C with gentle shaking. Retinas were washed with PBS and incubated with goat anti- mouse IgG AlexaFluor 488 (as the primary IgG stain to measure IgG extravasation) and goat anti-rabbit IgG AlexaFluor 546 (1:500, ThermoFisher) under identical conditions. Retinas were mounted onto Poly-Lysine coated slides (Tranjan) with the vitreous side facing upwards.

Retinal wholemounts were imaged at 20x magnification using the Dragonfly Confocal Microscope with the Sona-4.2B-6 sCMOS camera (Andor, Oxford Instruments). A thickness of 40 µm was maintained for all neuroretina sections, ensuring comprehensive visualization of the vascular

layers, GFAP-astrocytes, and Ibal-microglia. Fluorescence micrographs were captured with the following settings for emission wavelengths: IgG – 521/38 nm, 42.5 ms exposure, 24% laser intensity; Iba1 – 594/43 nm, 42.5ms exposure, 24% laser intensity; GFAP – 685/47 nm, 42.5ms exposure, 18% laser intensity. Identical imaging parameters and z-stack acquisition settings were applied. De-identified retinal images were collected as .ims files and batch converted to maximum projection.tiff files using FIJI software (ImageJ, US). Semi-quantitative analyses of GFAP and Iba1 immunointensities in collapsed z-stacks using ZEN Blue 3.3 Desk software provided insight into inflammation, encompassing all microglial layers of the neuroretina. The presence of extravascular IgG was first assessed in Imaris software (Oxford Instruments) on the 3D structure of the retina reconstructed from the z-stacks. The analysis was later corroborated on the max projection .tiff files using ZEN Blue 3.3 Desk software. The boundaries of IgG leakage in the tissue parenchyma were determined manually by two researchers blinded to animal experimental groups to ensure unbiased evaluation.

Statistical Analysis

Statistical analyses were performed in GraphPad Prism 9 (U.S.A). Data are represented as mean ± SEM and significance was considered at $P< 0.05$. The D'Agostino–Pearson omnibus was used to verify data normality. For normally distributed data, parametric one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) post-hoc test was employed for pairwise comparisons. Correlations were examined using Pearson's coefficient to analyse two associations: 1) between BBB integrity and cerebral inflammatory/oxidative markers, and 2) between cognitive outcomes and cerebral pathophysiological measures.

ADDITIONAL INFORMATION

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AUTHOR CONTRIBUTIONS

MM: Contributed to the study design, conducted comprehensive research, provided support in OCT imaging, performed data analysis and interpretation, and authored and edited the manuscript. JM: Contributed to the study design and results interpretation and provided primary manuscript edits. VL: Contributed to study design, provided expertise in animal maintenance, and contributed to manuscript edits. SM: Specialised in animal anaesthesia, collected OCT images, and participated in manuscript editing. MN: Assisted with sample collection, optimised immunofluorescence staining and analysis. EB: Contributed to study design, conducted preliminary literature review, and participated in manuscript editing. AS, AM, and HS: Equally contributed to manuscript editing. FC: Provided consultation and advice regarding the suitability of OCT imaging. RT: Provided oversight of the project, contributed to study design, assisted with data interpretation, and participated in manuscript editing. All authors approved the submitted version.

DATA AVAILABILITY STATEMENT

The data supporting this study are available upon request. Please contact Ryu Takechi at r.takechi@curtin.edu.au for access to the data.

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3.3 APPENDIX: EVALUATION OF CBD BIOAVAILABILITY IN LONG-TERM DIETARY ADMINISTRATION

3.3.1 Background

CBD's low oral bioavailability and susceptibility to oxidative degradation present considerable challenges in its pharmacological application (Millar et al., 2018). To address these issues, a pilot bioavailability study was completed before initiating the treatment study outlined in this chapter. The pilot aimed to assess the effectiveness of a novel encapsulation method alongside the co-administration of CBD with the transport-enhancing agent deoxycholic acid (DCA). The results are presented in Appendix 2 of this thesis as a published manuscript, of which I am a co-first author (Majimbi et al., 2021), entitled *"Sodium alginate microencapsulation improves the short-term oral bioavailability of cannabidiol when administered with deoxycholic acid."*

Scaling the encapsulation technique to fit the chronic treatment paradigm presented challenges, primarily in mass production and consistent distribution within the dietary chow. Therefore, further optimisation and characterisation of CBD microcapsules were not part of this PhD scope. Consequently, protocols for the manufacture, storage and animal feeding schedule of all treatment diets were modified to mitigate CBD's potential for photo-oxidative degradation.

3.3.2 Confirmation of CBD levels

Acknowledging the possibility of CBD diets being more susceptible to degradation than other treatments, I wanted to confirm the presence of CBD in db/db mice. Plasma samples and brain homogenates from db/db mice treated with CBD (50 mg/kg) and SAC+CBD (50 mg/kg and 75 mg/kg, respectively) were analysed using methods as described in our publication (Majimbi et al., 2021).

Quantitative analysis as shown in Table 3.1, confirmed measurable levels of CBD in the plasma and brains of treated db/db mice. Expectedly, CBD was below the level of quantification (LOQ) in diabetic db/db mice on control chow, the base AIN93M diet for all treatments. Importantly, chronic oral provision of CBD was well tolerated and appeared to have no impact on animal behaviour and welfare as compared to db/db mice on diets with or without treatment.

Table 3-1 CBD concentration in plasma (ng/mL) and brain samples (ng/g) from diabetic db/db mice following a 23-week dietary intervention with control chow, CBD (50 mg/kg) and SAC+CBD (50mg/kg and 75mg/kg, respectively).

	Plasma (ng/mL)			Brain (ng/g)		
	Control chow	**CBD**	**SAC+CBD**	**Control chow**	**CBD**	**SAC+CBD**
Number of samples	3	3	6	3	5	5
Mean	< LOQ	33.6	37.9	< LOQ	114	99
SEM	< LOQ	6.98	3.42	< LOQ	12.8	5.17

3.3.3 Considerations and Implications

The abundance of CBD observed in our study is comparable to a previous study utilising a voluntary administration technique of CBD (100 mg/kg for 5 days a week) for 6 months in a transgenic mouse model of spontaneous seizures (Dearborn et al., 2022).

When considering the pharmacokinetic profile of 'naked CBD' established in our acute oral gavage study with a 5 mg/kg dose (Majimbi et al., 2021), the increased systemic and CNS bioavailability of CBD in the present study may reflect a cumulative effect over time due to chronic CBD administration at higher dose (Millar et al., 2019). This sustained exposure is particularly relevant given the high first-pass metabolism and potential for photo-oxidative degradation of CBD. Furthermore, the small increase in plasma CBD levels when co-administered with SAC, while not statistically siginificant, indicates a possible interactive effect that merits further investigation. Whether SAC influences the metabolic stability of CBD, or its absorption from the gastrointestinal tract, or alternatively, affects the BBB permeability to enhance CBD uptake into the brain, remains to be elucidated.

Intriguingly, the plasma concentration detected after six months of administration, despite being higher than the Cmax of the acute study (Majimbi et al., 2021), is proportionally lower than might be expected, considering the fifteen-fold increase in dosage. This discrepancy could be due to a saturation of absorption pathways over the extended administration period. It is noteworthy that there may be limitations in pharmacokinetic prediction from acute to chronic administration paradigms. Future studies should aim to establish a clear relationship between dosage, treatment duration and the potential influence of co-administered compounds such as SAC. Moreover, future studies of CBD and all treatment agents utilised in this study, should consider the frequency of administration (ad libitum for our study) and its translatability to clinical contexts. The provision of dietary treatments to animals while group caged also presents challenges in accurate dosing that future studies should mitigate if possible. While group caging generally recommended, it is a confounder in assessing dietary intake of each animal. Further chemical characterisation of the treatment chow will be helpful to confirm changes in treatment potency to fully understand the implications of these results. Nevertheless, the presented data provides a compelling indication of the presence and potential therapeutic concentration of CBD in the CNS.

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CHAPTER 4. GENERAL DISCUSSION, LIMITATIONS AND FUTURE WORK

4.1 OVERVIEW OF KEY RESEARCH FINDINGS

This thesis confirmed that db/db mice show clinically relevant pathology of T2D, exhibiting metabolic dysregulation and systemic inflammation. Notably, these mice presented CNS anomalies associated with T2D, such as cognitive impairment and neurodegenerative thinning of the inner retinal layers. The research further confirmed that the brain and retina undergo parallel CNS pathophysiological insults, including BBB/BRB leakage, neuroinflammation and oxidative stress. These disease mechanisms may explain the significant association between retinal neurodegeneration and cognitive decline.

The therapeutic potential of SAC and CBD in alleviating T2D-related CNS complications is a significant and novel finding of this research. Both SAC and CBD, individually and synergistically, demonstrated neuroprotective effects by reducing astrocyte reactivity and microglia activation. Moreover, CBD was particularly effective in attenuating oxidative DNA damage. Notably, the combined SAC+CBD treatment showed a distinct vascular protective effect, with efficacy comparable to metformin in preventing BBB leakage. These therapeutic benefits correlated with improvements in memory function and retinal health, either matching or surpassing those observed with metformin treatment.

Interestingly, the effects of SAC and CBD on peripheral measures of diabetic metabolic syndrome were less pronounced, with no significant improvements in glycaemic control, or insulin resistance. This suggests a potential dissociation between metabolic control and neuroprotection, warranting further investigation. Therefore, this thesis underscores the need for a paradigm shift in the therapeutic management of CNS complications in T2D, extending beyond conventional glycaemic control.

This discussion chapter will explore how these findings contribute to the broader research on T2D and its associated complications. It will also critically assess the limitations of the current work and propose avenues for future research in both preclinical and clinical settings.

4.2 THE METABOLIC AND INFLAMMATORY FEATURES OF T2D PROMOTE CNS COMPLICATIONS

Before exploring the CNS complications linked to T2D, it was essential to assess the effectiveness of the db/db mouse model in replicating clinical T2D characteristics. Data from Chapters 2 and 3 demonstrated that db/db mice mimic early to moderate phenotypes of T2D, displaying elevated plasma glucose, insulin, triglycerides, and pro-inflammatory cytokines, concomitant with obesity. The progressive insulin resistance, validated through HOMA-IR and TyG index measurements, highlights the dual impact of insulin and triglycerides on disease progression (Laws et al., 2017; Selvi et al., 2021). Importantly, db/db mice did not exhibit extreme manifestations of T2D, such as substantial beta-cell loss leading to complete insulin deficiency (Galicia-Garcia et al., 2020). This alignment with the less severe, yet more common, presentations of T2D in humans underscores their relevance for studying population health outcomes. Furthermore, the detection of diabetogenic metabolites and inflammatory mediators in circulation serves as a predictor for both peripheral and central complications, as discussed in Chapter 1. By confirming hallmark features of T2D, this study not only strengthens the validity of the db/db model but also provides a foundation for investigating the underexplored central complications of T2D, including its association with cognitive decline.

4.3 COGNTIVE DYSFUNCTION: AN ESTABLISHED CNS COMPLICATION OF T2D

This research revealed cognitive deficits in db/db mice, initially evident in long-term aversive memory at 14 weeks and short-term episodic memory by 28 weeks. Consistent with prior studies, mild spatial learning and memory impairments emerged in db/db mice as early as 12-14 weeks, with notable exacerbation around 26-28 weeks (Ramos-Rodriguez et al., 2013; Yermakov et al., 2019). Similar trends in cognitive decline are evident in human studies, progressing from mild impairments to dementia-like pathology (Biessels et al., 2020). Our findings, supported by existing literature, suggest that distinct vulnerabilities within specific cognitive systems in T2D. The accelerated decline in both long-term and short-term memory functions likely stems from progressive neurodegenerative injury (Ortiz et al., 2022).

While db/db mice are valuable in research, they pose challenges for cognitive assessments due to their hypo-locomotive phenotype, potentially linked to obesity and polyneuropathy, though the exact cause remains uncertain (Yermakov et al., 2019). Moreover, other neurological comorbidities of T2D may confound memory tests in db/db mice. The model has been

successfully utilised in studies of depressive-like symptoms, potentially influencing behaviour in the NOR test (Ernst et al., 2013). Additionally, diabetic alterations in pain sensitivity (Galicia-Garcia et al., 2020; Nirenjen et al., 2023), may affect performance in the PA test, which relies on an aversive foot shock stimuli. Diabetic-induced deficits in visual function also warrant consideration, especially in cognition tests reliant on spatial cues (Sheskey et al., 2021). Thus, future studies should consider all CNS symptoms and their impact on cognitive measures.

Future preclinical investigations could expand the cognitive tests panel across all domains, including executive function, which is compromised in both T2D individuals and db/db mice (Black et al., 2018; Yermakov et al., 2019). Adopting a longitudinal study design or increasing the sampling rate and experimental timepoints would be beneficial. For instance, significant impairments in learning and memory in db/db mice at 17 weeks suggest further exploration of CNS alterations around this time point (Zheng et al., 2017). Enhancing animal cognitive experiments by exploring environmental effects, caging and enrichment, researcher interaction, and gut microflora following dietary intervention may prove useful. However, the limitations of preclinical research in recapitulating complex cognitive parameters require validation of all findings in clinical cohorts. Nevertheless, our findings offer valuable insights into the distinct impact of T2D pathology on memory functions.

4.3.1 Investigating neurodegeneration for diagnostic and treatment avenues for cognitive dysfunction

The established link between cognitive dysfunction and brain (Moran et al., 2013; Roy et al., 2020) underscores the importance of investigating neurodegeneration. The medial temporal lobe (MTL), housing the hippocampus and amygdala, is involved in both short-term and long-term memory functions (Čepukaitytė et al., 2023), and shown to be vulnerable to degeneration in diabetes (Ortiz et al., 2022). Our findings suggest MTL atrophy, as db/db mice displayed impaired long-term memory and spatial association capacity. Integrating neuroimaging studies of regional atrophy with cognitive assessments could enhance diagnostic accuracy. Unfortunately, MRI imaging is unlikely to be routinely employed for T2D diagnostics due to its high-cost, limited accessibility and implementation burden. Exploring retinal indicators of neurodegeneration offers a promising alternative, providing insights into cerebral atrophy and cognitive decline.

4.4 RETINAL NEURODEGENERATIVE PATHOLOGY: ASSOCIATION WITH COGNITIVE DYSFUNCTION IN T2D

4.4.1 Neurodegeneration in the inner retina in T2D

In Chapters 2 and 3, OCT imaging revealed a reduction in GCC thickness in db/db mice at 28 weeks, indicating a loss of RGCs. These findings align with Yang et al., (2015), who also observed retinal thinning at the same timepoint, confirmed through OCT and histological techniques. The study by Sohn et al., (2016) highlighted the early susceptibility of RGC cell bodies and axons in T2D, noting changes in the RNFL+GCL thickness in db/db mice as young as 10 weeks. Taken together, evidence suggests that retinal neurodegeneration is progressive and driven by neurovascular and inflammatory lesions. In human contexts, retinal thinning is increasingly recognised as an early marker of ocular pathology in T2D, with a causative link to the clinical onset of diabetic retinopathy (Lim et al., 2020; Sung et al., 2022).

As the output neurons of the retina, RGCs are crucial for visual function (Santos et al., 2017). RGC atrophy is linked to deficits in visual acuity and contrast sensitivity (Joltikov et al., 2017) and emergence of neurovascular abnormalities (Bronson-Castain et al., 2009). Moreover, the direct connection of RGCs to the brain *via* the optic nerve indicates that their degeneration signifies broader CNS implications (Pedersen et al., 2023; Ryan et al., 2023).

While our measurement of GCC thickness provides a robust marker of inner retinal changes, future studies could consider disaggregating the NFL, GCL, and IPL for a nuanced view of T2D pathology. Advancements in automated segmentation could further elucidated the characteristics of RGC loss. Implementing longitudinal OCT studies could enable both researchers and clinicians to track the progression of retinal changes in T2D and their neurocognitive ramifications.

The scope of this research did not extend to functional retinal tests such as the optokinetic nystagmus test, which could have provided insights into visual acuity and ocular motor responses in T2D. Incorporating a suite of electrophysiological measures and vision-centric assessments could enrich our understanding of functional outcomes of RGC loss. Moreover, complementing OCT with detailed *ex vivo* confirmation of RGC apoptosis, through assessment of caspase activity and assays like TUNEL, would add depth to our understanding of T2D-induced neurodegeneration. Diverse research methodologies could improve the translational

value of OCT metrics, guiding diagnostic and therapeutic developments for CNS health in T2D.

4.4.2 The relationship between retinal neurodegeneration and cognitive dysfunction in T2D

A notable finding in Chapter 2 was the significant correlation between retinal GCC thinning and memory impairments, suggesting a potential causative association with inflammatory and neurovascular lesions in the brain (Li et al., 2023; Sinclair et al., 2022). Interestingly, even mild retinal neurodegeneration corresponds to decreased stimulation of neurotrophic factors within the brain, corresponding to atrophy in regions such as the hippocampus and cortex (Garzone et al., 2023). This pathological relationship between retinal and brain neurodegeneration may also involve injury along the optic nerve and the disintegration of central visual pathways (Mendonca et al., 2020). While the mechanisms are not confirmed in this thesis, our data provides novel evidence highlighting the interdependence of retinal and brain health.

Further investigation into the complex relationship between retinal neurodegeneration and cognitive decline is warranted. While our research observed impairments in long-term memory prior to GCC thinning in db/db mice at 14 weeks, suggesting cognitive impairment may develop independently of retinal degeneration, longitudinal studies are needed to clarify these mechanisms and timelines. Additionally, exploring diabetes-induced changes in the visual system, particularly those affecting the geniculostriate pathway (Garzone et al., 2023), could provide further insights.

4.4.3 Comparing retinal and plasma biomarkers for cognitive dysfunction in T2D

This thesis delved into the potential of plasma metabolic and inflammatory biomarkers to reflect cognitive dysfunction in T2D. The findings suggest that the brain-retina pathological axis and GCC neurodegenerative thinning are more consistent indicators compared to peripheral markers like inflammatory cytokines or metabolic dysregulation. While previous studies have highlighted correlations between plasma markers such as HOMA-IR, glycated hemoglobin, CRP, and cytokines IL-1β, IL-6, and TNF-α (Zhao et al., 2018), ongoing research is needed with a broader array of markers including AGE/RAGE, c-peptide, TGF-β, as well as

serum oxidative (8OHdG) and glial activation markers (GFAP) (Biessels et al., 2020; Ehtewish et al., 2022).

Understanding the impact of systemic complications of T2D on cognitive dysfunction remains pivotal, especially peripheral pathogenic events may precede CNS complications. This avenue could offer a cost-effective and accessible means of assessing cognitive dysfunction in T2D, presenting a promising opportunity for future research.

4.5 CNS COMPLICATIONS AND SHARED PATHOPHYSIOLOGY IN T2D

4.5.1 BBB leakage: A Key Feature of T2D-associated Cognitive Dysfunction

This research advances our understanding of the role of BBB dysfunction as a key component of CNS pathology in T2D, focusing on the progressive nature of NVU impairment in the db/db model. Previous studies identified increased BBB permeability to small molecules in early-stage diabetic mice, evident at 8 weeks with 376 Da sodium fluorescein, and associated with subsequent cognitive dysfunction (Cai et al., 2021; Stranahan et al., 2016). With progression of the T2D phenotype in db/db mice at 16 weeks, the BBB remained intact against larger molecules, despite increased deposition of 150 kDa IgG complexes in the endothelial lumen (Liu et al., 2021). The work presented in Chapters 2 and 3 demonstrated the worsening of cerebrovascular pathology, with BBB leakage of IgG in brain parenchyme of db/db mice at 28 weeks, associated with glial activation, neuronal oxidative damage, and memory impairments. Indeed, IgG accumulation and extravasation cause disruptions to cerebral blood flow and enhance glial-mediated inflammation (Li et al., 2023). Therefore, our findings not only depict an advanced BBB pathology, they align with clinical findings connecting progressive hyperpermeability to cognitive decline (Sinclair et al., 2022; Starr et al., 2003).

Interestingly, our data showed no apparent IgG extravasation in diabetic db/db mice at 14 weeks, despite long-term memory deficits with concomitant glial and oxidative pathophysiology. Evidence suggests that brain capillaries are dysfunctional even prior to overt leakage, both driven and exacerbating the inflammatory and oxidative cascade in the CNS. Clinical studies support that dysfunction role of ‘intact’ capillaries as a significant vascular contributor to CNS complications in T2D (Musen et al., 2012). Alterations in cerebral perfusion have been identified in brain regions that are crucial for cognitive function, both in

T2D (Lasek-Bal et al., 2012) and Alzheimer's disease (Cantin et al., 2011). Accumulating evidence suggests that such vascular aberrations may represent a unifying pathology in neurodegenerative disorders (Shekhar et al., 2017). Notably, research by Duarte et al., (2023) reported described brain perfusion rates as 'sluggish' in individuals with T2D. Future studies should consider examining early, non-leakage vascular pathologies (endothelial AGE/RAGE, metabolic signalling, pericyte support, extracellular matrix composition, etc) in the CNS of T2D subjects with cognitive dysfunction. These may include characterisations of molecular changes in Understanding the mechanisms of cerebrovascular pathology prior to leakage is a critical area that may provide some insight into disease trajectory.

For a more nuanced assessment of NVU breakdown in T2D, future studies of BBB leakage should also include a range of experimental timepoints and tracers of varying sizes. It is also imperative to investigate vascular hyper-permeability during the pre-diabetic stages, as research has previously demonstrated BBB leakage in normoglycemic db/db mice as young as 3 weeks (Corem et al., 2019). These neurovascular aberrations are likely attributable to glycaemic-independent metabolic dysregulation that preferentially impacts glial cells within the CNS.

4.5.2 Glial Activation: Catalyst for Inflammation, Oxidative stress and NVU Breakdown in T2D-associated Cognitive Dysfunction

This research showed persistent glial activation in diabetic db/db mice evident by 14 weeks, suggesting its involvement may precede and drive overt BBB leakage. Reactive astrocytes, particularly at their end feet, mediate neurovascular uncoupling, inflammation, oxidative stress, and central insulin resistance, potentially leading to neurodegeneration and functional impairment (Hösli et al., 2022; Shen et al., 2023; Stephen et al., 2014). Similarly, chronically activated microglia promote neurotoxicity *via* secretions of inflammatory mediators (TNF-α, IL-6, IL-1β, CXCL10) and toxic metabolites (AGEs, FFAs, etc.), in addition to increased phagocytic activity (Sheikh et al., 2022; Stranahan et al., 2016). Co-activation of glial cells suggests a significant diseased state, posing an elevated risk of irreversible CNS damage (Shigemoto-Mogami et al., 2018).

The interplay between glial cells is complex and multifaceted. For instance, astrocytes secrete chemokines, including CX3CL1 (fractalkine), which acts on CX3CR1 receptors primarily

found on microglia. Under healthy conditions, microglia rely on fractalkine/CX3CR1 signalling to dampen inflammatory responses, mediate synaptic pruning, and maintain cognitive function (Vargas-Soria et al., 2023). This mechanism is defective in T2D, leading to synaptic stripping in the hippocampus (Hao et al., 2016; Vargas-Soria et al., 2023). Therefore, this is a mechanistic pathway of glial activation that requires further investigation in T2D. Disruptions in astrocytic internal regulation also drive glial activation more broadly, and concurrent neuronal dysfunction. For example, astrocytes in T2D exhibit impaired glutamine-glutamate cycling and gliotransmission, contributing to reduced synaptic strength and plasticity in the hippocampus and cortex, which correlate with cognitive impairment in T2D (Fried et al., 2017; Trudeau et al., 2004). In animal models, neuronal damage from excitotoxicity has been associated with GFAP-astrogliosis and has been shown to lead to microglial activation, along with diminished hippocampal synaptic plasticity and deficits in spatial and fear memory (Amin et al., 2013; Vargas-Soria et al., 2023).

Moreover, it has been demonstrated that the interplay between activated microglia and astrocytes contributes to BBB dysfunction via pro-inflammatory and oxidative pathways that compromise cell survival (Dresselhaus & Meffert, 2019). For example, dysfunction in the MAPK pathway leads to activation of hypoxia-inducible factor 1-alpha (HIF-1α), which in turn upregulates ROS and growth factors (such as VEGF) that amplify neurovascular hyperpermeability and neovascularisation (Liu et al., 2021; Mei et al., 2018). While outside this scope, molecular characterisations in reactive gliosis are needed, including the aberrations between in the NFκB/Nrf pathway and the NLRP3 inflammasome that are crucial for sustained activation. This molecular evidence we have thus far supports the conclusion drawn from our findings in Chapters 2 and 3 that astrocyte reactivity and microglial activation contributed to cognitive impairment in diabetic db/db mice through corruption of metabolic and inflammatory pathways, leading to vascular compromise that exacerbates neurodegeneration.

Our findings of elevated cerebral oxidative DNA damage at 14 weeks, which was sustained and localised in the hippocampus at 28 weeks, suggest that T2D induces changes in the CNS at the genomic level. Although this damage was not specifically confirmed within astrocytes or microglia, the correlation between oxidative stress, glial activation, and BBB leakage suggests a potential link. Importantly, oligodendrocytes and endothelial cells are also highly vulnerable to oxidative DNA damage, and their role in this context should not be overlooked (Luc et al., 2019). Future studies should aim to localise the site of oxidative damage to better

understand its origin and implications. Moreover, the vulnerability of nucleic acids to oxidative stress encompasses a range of lesions beyond 8OHdG, such as double-strand breaks. Therefore, future research should widen the scope to include a comprehensive assessment of nucleic acid damage and its potential repercussions on gene expression and genomic integrity in T2D.

Finally, the impacts of oxidative stress on proteins and lipids, and the activities of key enzymes in redox balance. These may include measures of protein carbonylation and lipid peroxidation, as well as the activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase, alongside the levels of reduced and oxidised glutathione (GSH/GSSG ratio). To better understand the pathophysiological consequences of reactive glia cells in T2D more broadly, it's essential to assess the CNS' proinflammatory milieu (cytokines/chemokines, AGEs, FFAs, ATP, glutamate, etc). Nevertheless, our research findings provide evidence that abnormal glial activation and oxidative stress are core disease mechanisms impacting the NVU, working in concert to promote neurodegeneration that disproportionately impacts memory centres in T2D.

4.5.3 Pathological parallels in the diabetic retina: BRB leakage and glial activation

In the diabetic db/db mice retina, pathological changes paralleled those observed in brain tissue, encompassing BRB leakage, astrogliosis, and microglial activation. It is posited that the CNS complications associated with T2D arise from shared inflammatory and neurovascular degenerative mechanisms (Sinclair et al., 2022). Our findings have been corroborated in various models of T2D, including in non-human primates, where elevated GFAP-astrogliosis and Iba1-microglial activation corresponded to GCC degenerative thinning in retinal OCT imaging (Xia et al., 2021). Additionally, the authors reported "exudation and effusion in the blood vessels," providing evidence for neurovascular corruption under inflammatory conditions (Xia et al., 2021, p.1).

At the cellular level, disruptions in the BRB and a proliferation of microglia within the inner retina contribute to the apoptosis of retinal ganglion cells RGCs, as observed in recent findings (Hachana et al., 2020). Notably, retinal microglia demonstrate a unique proximity to the BRB, with structural and functional dynamics that may surpass those in cerebral tissue (Mills et al., 2021). Such retinal sensitivity to metabolic and immune disturbances may explain the prevalence of retinopathy as the most common complication of T2D (Sinclair & Schwartz,

2019). Ongoing research should further detail the microglial dynamics within the diabetic retina, confirming their detectability as hyperreflective foci in OCT imaging (Lee et al., 2018).

Our study, presented in Chapter 3, indicated a reduced incidence of BRB leakage in diabetic db/db mice compared to prior research (Byrne et al., 2021; Cheung et al., 2005). However, significant differences in the molecular sizes of the tracers used and experimental aged time points (12 weeks – 15 months) call for a careful interpretation of these results. Future investigations could employ tracers of varied molecular sizes to study leakage, alongside cerebral assessments, to enhance reproducibility and comparability of results.

Similar to cognitive outcomes, examination of the diabetic retina in mice should always be corroborated in human studies where possible. Species-specific variances in structure and function must be examined critically, particularly when extrapolating rodent data to human contexts (Fernández-Albarral et al., 2022). One such example is that astrocytes in the retina display notable species-specific structural variations. Humans possess astrocytes that are both elongated (NFL) and star-shaped (GCL), whereas rodents exhibit only stellate-shaped astrocytes that lie across the retina's surface (Sheskey et al., 2021). Future investigations of glial cell morphology and functions in rodents should be validated with donor tissue to ensure relevance to human retinal health and diseases. Nevertheless, the observed congruence in BBB/BRB breakdown and glial activation across the CNS, including the symbiotic relationships of neural tissues, provide strong evidence for a shared pathophysiology in T2D.

4.6 SAC AND CBD ARE PROMISING NEUROPROTECTIVE AGENTS IN T2D

4.6.1 Neuroprotective effects of SAC and CBD in the diabetic brain

Dietary supplementation with SAC and CBD showed remarkable potential in enhancing cognitive function and mitigating cerebral pathophysiology in diabetic db/db mice. Previous studies indicated that these natural compounds could protect against neurodegenerative disease and central metabolic dysregulation, although the disease models were not entirely representative of T2D pathology (Brook et al., 2019; Kosuge, 2019).

Mechanistically, SAC and CBD readily cross the BBB/BRB and exert therapeutic effects by modulating glial activation, regulating pathways such as NF-κB and Nrf2, scavenging pro-

oxidant species, and elevating neurotrophic factors (Colín-González et al., 2015; da Silva et al., 2018; Vallée et al., 2017). Additionally, CBD's interaction with the endocannabinoid system and GPCRs contributes to the wide-ranging neuroprotective effects reported in various disease models (Peng et al., 2022).

In Chapter 3, the mice treated with SAC and CBD showed a complete prevention of the cognitive decline in db/db mice. SAC has been shown to ameliorate deficits in spatial learning and memory recognition by mitigating glial activation and protecting against DNA damage (Javed et al., 2011; Zarezadeh et al., 2017; Zeinali et al., 2018). Meanwhile, CBD has been reported to alleviate episodic memory deficits and attenuate BBB leakage, microglial activation, astrocyte reactivity and neuronal atrophy in the hippocampus (Esposito et al., 2011; Fagherazzi et al., 2012; Mecha et al., 2013).

This research is the first to demonstrate the efficacy of long-term treatment with SAC and CBD, presenting significant therapeutic potential for individuals with T2D. Future research could build upon our findings with longer treatment regimens to characterise the effects of SAC and CBD treatment on dementia-like phenotypes and advanced CNS pathology in T2D.

4.6.2 Neuroprotective effects of SAC and CBD in the diabetic retina

Chapter 3 also showed for the first time the neuroprotective effects of SAC and CBD, either alone or in combination, against GCC thinning in T2D. Treatment with the SAC+CBD combination notably prevented the thinning of the total neuroretina and the INL, which are subject to age-associated alterations independent of T2D. This suggests a potential synergistic effect in preventing age-related retinal changes. However, the observed increase in ONL thickness in diabetic mice receiving SAC+CBD raises questions, as reports on ONL thickness changes in T2D are inconclusive (Chen et al., 2016; Ishibashi & Tavakoli, 2020; Vujosevic & Midena, 2013). Further research is warranted to clarify the effects of aging and diabetes on the ONL, along with the impact of SAC and CBD treatments on various neuronal populations that are arranged across the 10 layers of the retina.

This thesis reported positive trends for the effects of SAC and CBD on BRB leakage risk and microglial activation, consistent with previous studies (Chao et al., 2014; El-Remessy et al., 2006). Given the limited research on SAC and CBD in the retina, this study provides valuable

preliminary retinal data supporting the targeting of glial and vascular components markers, thus substantiating the comprehensive neuroprotective effects of these compounds across both neural tissues.

4.6.3 Evaluating SAC+CBD combination treatment in T2D-associated CNS complications

The central hypothesis of Chapter 3 posited that a combination of SAC+CBD would offer a synergistic protection against CNS damage caused by T2D, building upon the well-documented individual effects of these compounds. Our study revealed that SAC+CBD co-treatment improve BBB integrity with greater efficacy than monotherapy groups. Existing research indicates that both SAC and CBD influence the expression of vascular adhesion factors, fibrotic markers and proteins like VEGF, which play roles in BBB/BRB permeability and neurovascular cell death (El-Remessy et al., 2006; Zeinali et al., 2018). It is possible that the apparent synergism of SAC+CBD combination treatment in vascular protection is due to their modulation of endothelial dysfunction. To better understand this treatment effect, future studies could examine a broader spectrum of vascular measures to provide deeper mechanistic insights into the potential synergistic effects.

Moreover, the current study suggests that SAC+CBD treatment may possess particularly strong antioxidative properties, even though CBD alone also markedly prevents DNA oxidation. This observation aligns with research proposing antioxidant regeneration and metal chelation as potential mechanisms in the synergistic effects of nutraceutical compounds (Leena et al., 2020).

Surprisingly, the efficacy of SAC+CBD treatment in glial modulation and GCC preservation did not significantly exceed that of monotherapies. Notably, monotherapy demonstrated unexpectedly superior results in enhancing memory retention. This could be attributed to a potential ceiling effect, where the maximal therapeutic impact is attained with single agents. It's also possible that the complex therapeutic actions of SAC and CBD do not synergise as initially thought (Colín-González et al., 2015; da Silva et al., 2018; Vallée et al., 2017). Indeed, the exploration of co-treatment with SAC+CBD is in the very early stages. Given that monotherapies show superior memory preservation, further work is needed on the cognitive effects of both molecules in animals and humans prior to the endorsement as nootropic agents.

Finally, investigations in naturally-derived therapies should consider their effects on various mechanisms of cognitions that include neurotransmitter regulation and neural connectivity, which are compromised in T2D associated memory decline (Alvarez et al., 2013; Fried et al., 2017; Trudeau et al., 2004).

4.7 COMPARING NEUROPROTECTIVE EFFICACY OF METFORMIN AGAINST SAC AND CBD IN T2D

Metformin, as the first-line treatment for T2D, has been considered for its neuroprotective potential. Yet, the evidence remains inconclusive. Unlike SAC and CBD, metformin did not show significant cognitive benefits in db/db mice, a finding that is both supported and refuted by recent clinical studies (Antal et al., 2022; Zhang et al., 2022).

Despite its limited impact on cognition, metformin exhibited comparable effectiveness to SAC and CBD formulations in mitigating vascular leakage, neurodegenerative GCC thinning and microglial activation in both neural tissues. However, a concerning trend emerged with metformin's amplification of oxidative DNA damage. Previous reports suggest that metformin has the potential to intensify CNS pathophysiological processes, including the production of ROS (Picone et al., 2015). Yet, there is a much larger body of evidence that metformin has anti-oxidative and neuroprotective actions (Karami et al., 2023), which would place our findings in the minority of studies that observe a worse effect with metformin.

The variable physiological effects of metformin, especially when compared with SAC and CBD, suggest that it may not offer as complete CNS protection. Studies using HFD mice and models of T1D and Alzheimer's disease have shown varied neuroprotective responses to metformin (da Silva et al., 2021; Mamo et al., 2019; Pilipenko et al., 2020), but differences in experimental designs make it difficult to draw direct comparisons. Future investigations could consider different dosages of metformin and a wider range of cognitive, inflammatory and oxidative indicators to clarify its effectiveness in addressing CNS complications associated with T2D.

4.8 STUDY LIMITATIONS AND FUTURE RESEARCH OPPORTUNITIES

This research has shed light on the relationship between T2D and CNS implications, however it is not without limitations. This section will primarily address the underexplored limitations

of this research candidacy, which present exciting avenues for future impactful work alongside the recommendations already discussed throughout this thesis.

4.8.1 Model Limitations

The db/db mouse model, known for its pronounced leptin signalling defects, might not perfectly replicate human T2D phenotypes, particularly due to severe disruptions in leptin-mediated pathways that can affect neuroglial and synaptic functions (Dilworth et al., 2021). Given that typical T2D manifestations do not include life-long leptin resistance, future work should always corroborate animal studies in a clinical cohort.

4.8.2 Metabolic Biomarkers

The use of postprandial instead of fasting blood metabolic biomarkers might restrict how our findings can be applied within the typical diagnostic framework for T2D. Moreover, this study's scope did not extend to verifying metabolic dysregulation and fluctuations in pro-inflammatory cytokines within the CNS—key factors that could corroborate the diabetic influence on cognition. A deeper understanding of the metabolic and inflammatory milieu of the CNS could shed light on the processes leading to glial cell activation and subsequent breakdown of the BBB/BRB. To substantiate and expand upon our research findings, future research should use fasting blood for peripheral assessments and compare with corresponding CNS outcomes.

4.8.3 Changes in Functional Connectivity and Cognitive Dysfunction

Neural connectivity alterations are increasingly recognised as early indicators of cognitive decline and cerebrovascular disease in T2D, potentially precipitating or aggravating neurodegenerative processes (Biessels & Reijmer, 2014; Zhang et al., 2016; Zhou et al., 2010). Expectedly, these alterations were most pronounced within regions governing learning and memory function (Liu et al., 2020). Abnormal connectivity has also been identified in the pre-diabetic stage, and its prevalence increased with progression of insulin resistance (Lopez-Vilaret et al., 2022). The investigations of connectivity were outside the scope of this research, and yet they present exciting opportunities for both imaging and functional studies in mice models and clinical studies. Given the Our research did not delve into the diabetic changes in brain synaptic activity and connectivity of memory-associated neural circuits. Given that we

did not investigate the functional consequences of GCC thinning, there are notable knowledge gaps in understanding of the overall CNS pathological environment. Future research could elucidate the interplay between cerebral and retinal functional connectivity in T2D in both human and animal studies.

4.8.4 Molecular Insights and Therapeutic Potentials

When considering therapeutic interventions, this study has already identified gaps in molecular characterisation. Therefore, in-depth pharmacokinetic and pharmacodynamic analyses of SAC and CBD would be informative in understanding interactive effects. Additionally, investigating the influence of these compounds on peripheral inflammation could yield valuable information, as their impact on metabolic parameters within this study was limited. Given that SAC and CBD exhibit entourage effects when administered with other cysteines and cannabinoids (Brook et al., 2019; Tsai et al., 2011), respectively, it may be informative to compare their effects as isolates and as part of a cocktail of naturally occurring compounds. Exploring SAC and CBD's role as adjunct therapies to established anti-diabetic medications may also be worthwhile.

4.8.5 Dosage Translation from Preclinical Models to Clinical Application

A constraint for translational medicine research is the use of dosages in animal models that may not correspond to human therapeutic ranges. Our research utilised doses of SAC, CBD, and metformin supported by literature for their therapeutic potential (De Oliveira et al., 2016; Dearborn et al., 2022; Iffland & Grotenhermen, 2017; Zarezadeh et al., 2017; Zeinali et al., 2018). However, when adjusting with allometric scaling, these doses appear disproportionately high for humans (Chandel et al., 2016; Kodera et al., 2002; McCartney et al., 2020). There is a risk that the biological responses observed in animal models may not be translatable at lower, clinically relevant doses in humans, leading to potential misinterpretation of a drug's efficacy and safety (Chandel et al., 2016).

To enhance translational validity, future research should consider dose-response studies in db/db mice. Detailed pharmacokinetic and pharmacodynamic studies are also recommended to understand how drugs behave at varying doses, thereby enabling more precise dosing guidelines for human trials. Nevertheless, this thesis provides a significant contribution to the

understanding of neuroprotective therapeutics in T2D and presents an extensive approach for the refinement of experimental methodologies.

4.9 RESEARCH SIGNIFICANCE

This research marks a significant contribution in our understanding of T2D, particularly regarding the pathological connection between metabolic dysfunction and cognitive decline. The integrated approach to diagnosing and treating T2D's CNS complications is often overshadowed by the predominant focus on managing peripheral symptoms. By establishing retinal thickness as a reliable biomarker for cognitive decline in T2D, this thesis fills a crucial gap in predictive diagnostics while advocating for the clinical utility of OCT in routine screenings.

The effectiveness of SAC and CBD in mitigating CNS complications provide compelling evidence for neuroprotection, exceeding the benefits of standard treatments like metformin. These therapeutic agents, potentially formulated as pharmaceutical supplements, offer a practical treatment avenue that can be easily integrated into community health initiatives. Equally significant, this work lays a foundation for further investigations into T2D's complications. Through ongoing research and eventual clinical trials, the insights gained here have the potential to significantly enhance quality of life.

4.10 CONCLUDING REMARKS

In conclusion, this thesis shed light on the memory impairment in T2D and identified retinal neurodegenerative as a potential surrogate marker for cognitive decline. Addressing the pathological mechanisms that lead to altered brain and retinal health is critical. The neuroprotective potential of SAC and CBD, individually and in combination, show promise in attenuating the risk for CNS complications in the db/db model T2D. Notably, SAC and CBD's effects in inflammatory modulation and cognitive support appear to exceed metformin. These key findings, with ongoing research, will address significant challenges for those suffering with T2D's CNS complications.

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APPENDIX 1: CBD MICROENCAPUSLATION PILOT STUDY

The following published article represents a significant research output, although it doesn't directly answer my PhD research objectives.

PLOS ONE

RESEARCH ARTICLE

Sodium alginate microencapsulation improves the short-term oral bioavailability of cannabidiol when administered with deoxycholic acid

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Abstract

Background

Cannabidiol (CBD) confers therapeutic effects in some neurological disorders via modulation of inflammatory, oxidative and cell-signalling pathways. However, CBD is lipophilic and highly photooxidative with low oral bioavailability in plasma and brain. In this study, we aimed to design and test a CBD microencapsulation method as a drug delivery strategy to improve the absorption of CBD. Additionally, we evaluated the brain uptake of CBD capsules when administered alongside capsules containing a permeation-modifying bile acid, deoxycholic acid (DCA).

Methods

Microcapsules containing either CBD or DCA were formed using the ionic gelation method with 1.5% sodium alginate formulations and 100 mM calcium chloride. C57BL/6J wild type mice randomly assigned to three treatment groups (3–4 mice per group) were administered CBD in the following preparations: 1) CBD capsules, 2) CBD capsules + DCA capsules and 3) naked CBD oil (control). To assess the short-term bioavailability of CBD, plasma and brain samples were collected at 0.3, 1 and 3 hours post administration and CBD levels were analysed with liquid chromatography mass spectrometer.

Results

We produced spherical capsules at 400 ± 50 μm in size. The CBD capsules were calculated to have a drug loading of 2% and an encapsulation efficiency of 23%. Mice that received CBD capsules + DCA capsules showed a 40% and 47% increase in CBD plasma

gifts from the company. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing interests: The study was financially supported by Zelira Therapeutics Ltd. This does not alter our adherence to PLOS ONE policies on sharing data and materials. The authors would like to declare the following patents/patent applications associated with this research: "Cannabinoid Encapsulation Technology", Patency number 2020904057.

concentration compared to mice on CBD capsules and naked CBD oil, respectively. Furthermore, the CBD capsules + DCA capsules group showed a 48% and 25% increase in CBD brain concentration compared to mice on CBD capsules and naked CBD oil, respectively. In mice treated with CBD capsules + DCA capsules, the brain CBD concentration peaked at 0.3 hours with a 300% increased availability compared to CBD capsules and naked CBD oil groups, which peaked at 1 hour after administration.

Conclusions

The microencapsulation method combined with a permeation enhancer, DCA increased the short-term bioavailability of CBD in plasma and brain.

Introduction

Cannabidiol (CBD) is a potent non-psychoactive constituent in marijuana (*Cannabis sativa)* with no reported intoxicating effects unlike tetrahydrocannabinol (THC) [1]. CBD binds to receptors CB1, CB2 and 5HT1A to modulate cellular activity and inhibit excitotoxicity [2]. The anti-inflammatory and antioxidant properties of CBD have been reported across numerous pathologies, including neurodegenerative and metabolic diseases. Studies show that repeated administration of CBD may be neuroprotective in animal models of Alzheimer's disease via decreasing microglial activation and attenuation of memory deficits [3]. Furthermore, activation of the endocannabinoid system has been shown to preserve the cerebral capillary endothelium that forms the blood brain barrier (BBB) [4, 5] and exert therapeutic effects in animal models of diabetes [6].

CBD is highly lipophilic, sensitive to light and largely broken down in the duodenum resulting in extremely low oral bioavailability in plasma and tissues (approximately 6% and 1%, respectively) [7]. Whilst chronic usage of CBD is clinically well tolerated, its lability poses limitations for use in research and adaptation as a pharmacotherapy [8]. Therefore, emerging studies have focused on encapsulation techniques that function as a vehicle for CBD [9].

Sodium alginate is an extract from brown algae that is biocompatible, hydrophilic, non-toxic and readily available for human use. The alginate cross-links with multivalent ions like Ca^{2+} to form stable hydrogel polymers under mild aqueous conditions [10]. Thus, calcium alginate microcapsules provide a 'physical' barrier for volatile drugs such as CBD against exposure to light and air in pharmacological protocols. Previously, our studies have demonstrated that sodium alginate microcapsules significantly increase the brain uptake and associated neuroprotective effects of probucol—the highly lipophilic, antilipidemic drug [11]. Subsequent studies of ours confirmed that microencapsulation improves therapeutic efficiency of lipophilic drugs by protecting against degradation due to low stomach pH and efflux protein activity in the intestinal epithelium [12, 13].

The use of absorption enhancers is yet another method of improving bioavailability in drug delivery research. Deoxycholic acid (DCA) is a metabolite of chenodeoxycholic acid, a primary bile acid made from cholesterol in the liver in humans [14]. Bile acids have amphiphilic properties and primarily function as surfactants in the body. DCA is among several secondary bile acids to be utilised in pharmacokinetic research as a permeation-modifying biomolecule [15]. Specifically, lipophilic drugs with low intestinal dissolution rates have shown improved absorption when administered with DCA [16]. Bile acids promote aqueous solubility and increase fluidity of phospholipid membranes, making them ideal candidates for BBB

permeability experiments [16]. A report from our lab showed that co-encapsulation with a DCA variant improved the targeted-delivery effects of probucol [12]. However, there is a relative paucity of studies focusing on the permeation effects of DCA on CBD absorption. The fundamental challenges of administering DCA alongside a labile drug such as CBD with minimal exposure to the environment and impost on experimental animals have yet to be resolved.

In this study, we utilised the established sodium alginate microencapsulation method to develop CBD capsules and assess the abundance of CBD in plasma and brain samples of wild-type mice. Furthermore, we administered CBD capsules alongside DCA capsules to examine the blood-to-brain kinetics of CBD in conjunction with a permeation enhancer. The study captured CBD absorption at three time points leading up to- and immediately post its peak concentration to elucidate the short-term effects of our drug excipients. The findings of this study may provide a potential avenue for improving the penetrance of CBD in models of neurodegeneration.

Materials and methods

Materials

CBD (14.5% solubilised extract in Miglyol 812N, a medium chain triglyceride carrier oil) and Miglyol 812N were kindly gifted by Zelira Therapeutics, (Perth, Western Australia). Medium Viscosity Sodium Alginate (MVSA) (≥2,000 cP, 2% (25˚C)), deoxycholic acid (DCA) and Calcium Chloride anhydrous (98%) were purchased from Sigma-Aldrich, (St Louis, MO, USA). Formulations were made up in HPLC grade deionised water.

CBD and DCA formulations

Solutions containing 1.5% MVSA in 80 mL of HPLC-grade deionised water were mixed overnight. The CBD formulation was made by adding 800 μL of Miglyol 812N containing 109.24 mg CBD to the MVSA solution while protected from air and light and mixed for 3 days. The DCA formulation was made by adding 10 mg of DCA to a separate MVSA solution and mixed overnight. The MVSA solution and drug formulations were mixed at the same speed at room temperature. The formulations were prepared according to a protocol developed in-house and published previously [17, 18]. The aforementioned protocol has been optimised through a series of experiments for rheology, fluid dynamics and colloidal dispersion electrokinetic (Zeta potentials) to ensure total emulsification of the drug content into the MVSA solution.

CBD and DCA encapsulation

The CBD and DCA formulations were encapsulated immediately after emulsification using the gelation technique with the vibrating Encapsulator B-390 (BUCHI Labortechnik, Switzerland). The encapsulation protocol was adapted from previous studies in our group [11], with minor adjustment of the capsulation conditions: frequency range of 2000 Hz and air pressure at 950 mbar through a 200 μm nozzle with a flow regulating valve set at 2 rotations from the tightest starting point.

Prepared formulations were projected into the 100 mM $CaCl_2$ hardening bath, which stirred with a mild vortex, at a flow rate of 5 mL/minute and formed spherical microcapsule beads. After 10 minutes in $CaCl_2$, microcapsules were sieved, rinsed with deionised water and dried with a paper towel patted under the strainer. They were placed on a petri dish, covered and dried completely at 37˚C for 2.5 days. Microcapsules were analysed and used for experimentation within 48 hours of drying.

Encapsulation efficiency

The CBD loading and capsule size were determined by using HPLC Prominence (Shimadzu LC-20AT liquid chromatographer, SIL-20A autosampler and SPD-20A-UV/Vis detector (Japan)) and particle analyser (Zetasizer 3000 HS and Mastersizer 2000, Malvern Instruments, Malvern, UK), respectively according to an established HPLC protocol from our lab [12] with some modifications to account for the known effects of oil on microencapsulation [19]. Briefly, 5 mg of microcapsules were agitated and suspended in PBS (pH 8.5) for 20 h at room temperature and centrifuged for 15 min at 13200 rpm at 10˚C. The supernatant was collected and diluted with Mobile Phase Mixture (ACN:Water = 75:25). Encapsulation efficiency was determined with published formulae summarised below as the relationship between theoretical and confirmed CBD loading within the microcapsules [13].

$$Drug\ loading\ (\%) = \frac{CBD\ in\ microcapsules\ (mg)}{Weight\ of\ microcapsules\ (mg)} \times 100$$

$$Encapsulation\ efficiency\ (\%) = \frac{Drug\ content}{Theoretical\ content} \times 100$$

In vivo animal experiments

Healthy C57BL/6J wild type mice obtained from Animal Resources Centre, WA were group housed in a temperature-controlled laboratory on a 12-hr light/dark cycle with standard chow and water provided *ad libitum* (14 months, female, average weight 36±3 g, n = 45). The mice were fasted overnight prior to CBD administration. Experiments were conducted according to approved animal ethics protocol (Curtin Animal Ethics Committee, approval no. ARE 2018–19).

Mice were restrained and orally administered 5 mg/kg weight CBD in oil by itself (naked CBD oil), in capsulation (CBD capsule), or in capsulation with additional 4 mg/kg weight DCA capsules (CBD capsule + DCA capsule). CBD capsules were combined with DCA capsules at the time of administration. The drug/s were mixed in raspberry jam, which improved palatability. The same amount of raspberry jam (mg) was given to all mice, including the naked CBD oil group to limit treatment variability. Prior to sacrifice, mice were anesthetised with isoflurane gas and blood was obtained via cardiac puncture into EDTA-coated tubes. Mice were euthanised by cervical dislocation and the brain was collected and snap frozen immediately at 0.3 hours, 1 hour, and 3 hours post-administration. Four mice were used per time point per group, except for the Capsule and Naked groups at 1 hour, where three mice were used. This was determined based on previous similar studies of CBD pharmacokinetics [9, 25]. Plasma was collected by centrifuge (2500 g, 10 minutes, 4˚C). Samples were stored at -80˚C until analyses.

Measurement of CBD concentration

The concentration of CBD in plasma and brain tissue was determined using the protocol described previously [20]. Briefly, plasma samples were thawed and 50 μL were added to 100 μL of acetonitrile containing deuterated internal standards. Brain samples (500 mg) were homogenised in methanol (500 μL) using a Tissue-Lyser with a 3 mm steel ball bearing, the samples were then centrifuged and 50 μL of homogenate was added to 100 μL of acetonitrile containing deuterated internal standards. Both plasma and brain samples were vortexed, then centrifuged and the supernatant was transferred to a vial and injected onto the LCMS system (Shimadzu 8060, Shimadzu, Australia). A Kinetex Biphenyl column (50 ×3 mm, 2.6 μm) using a gradient of 0.1% formic acid and acetonitrile was used for the analysis. The calibration curve ranged from 0.5–500 ng/mL with a limit of quantitation of 0.5 ng/mL.

Pharmacokinetic and statistical analysis

The results are expressed as mean and where applicable, the standard error of mean (SEM) is provided. Graphpad Prism v7 (Graphpad, Inc., USA) was used to generate concentration-time graphs on linearized $\log_{10}$ scale and calculate area under the concentration curve (AUC). Statistical significance between treatment groups was determined using one-way ANOVA with significance at $p < 0.05$.

Pharmacokinetic parameters were approximated using established models in Microsoft Excel using the add-in program PKSolver (Microsoft Excel 2010) and are expressed below as median [21]. These included terminal half-life ($t_{1/2}$), maximum concentration (C_{max}), time to reach Cmax (t_{max}), area under the concentration curve from zero to a certain time (AUC_{0-t}), and from zero to observed infinity ($AUC_{0\text{-}inf\_obs}$).

Results and discussion

To improve the oral bioavailability of CBD, we assessed the pharmacokinetic profile of CBD capsules as a standalone treatment and in combination with capsules containing the permeation enhancer, DCA; compared to naked CBD oil. The microencapsulation technology utilised in this study was pioneered by our laboratory and has been adopted for enhanced lipophilic drug delivery in varied preclinical models of metabolic disorders [11, 15].

Characterisation of capsulated CBD

The formulation that generated the most optimal, reproducible microcapsules was at 1.5% MVSA and 1% CBD. The microcapsules were spherical, and their size was 400±50 μm immediately post drying. The CBD capsules were white and their appearance remained consistent for 30 days at room temperature; followed by gradual discoloration with the capsules appearing brown, rough and shrived. Following the desiccation process, over 98% of capsulated CBD remained intact after exposure to the ambient air and light for 72 hours, indicating marked protection of CBD by the MVSA encapsulation. This data suggests that the encapsulation protocol may provide protection of CBD from exposure to ambient air, potentially improving shelf-life stability. Further tests are required to extensively characterise the physical properties and stability of the CBD capsules [22].

Calculated encapsulation efficiency of CBD was 23±1.2% and CBD loading was estimated as 2.05±0.10%. Drug loading was comparable to previous studies [23]. The encapsulation efficiency remained relatively low despite multiple optimisations. A recent study by Wagle et al. [23] that utilised a similar encapsulation protocol on probucol reported a drug loading of approximately 2% but with an encapsulation efficiency as high as 92%. The drug used in the aforementioned study was in powder form, which may have been a major determinant of the encapsulation efficiency. Further investigations will be needed to evaluate the effects of the oil content of our CBD preparation.

Pharmacokinetics of capsulated CBD

The present study used a dosage of 5mk/kg, which is lower than the typically administered dosage of 20 mg/kg in previous studies [24, 25]. The small dosage was chosen to mitigate the low encapsulation efficiency, whilst yielding plasma and brain concentrations that could be detectable for this short-term study [26]. CBD pharmacokinetics, including C_{max} and AUC in plasma and brain samples have been demonstrated to be dose-dependent in both animal [27] and clinical studies [8], making it difficult to compare these results with published literature.

Fig 1 illustrates the mean plasma and brain concentrations of CBD in mice administered with 1) CBD capsules, 2) CBD capsules + DCA capsules and 3) naked CBD oil–along with the

corresponding area under curve (AUC) graphs. Data was accrued at three time points over 3 hours to focus on the acute bioavailability of CBD encapsulation. The short-term effects of CBD in mice are of interest to our lab due to their observed fast metabolic rates. For example, the plasma elimination half-life of injected CBD is shorter in mice at 4.5 hours [24] compared to humans at 24 hours [28]. Short-term pharmacokinetic studies, while not common, have been published with a focus on brain and plasma availability. A recent study looked at the penetrance of phytocannabinoid acids (CBDAs) in mice at 5 timepoints up to 2 hours post intraperitoneal injection [29]. Unsurprisingly, the study reported poor brain absorption of CBDAs, which limited the therapeutic outcomes of the drugs. This study aimed to optimise short-term pharmacokinetics to improve the neuroprotective effects of CBD.

At 0.3 hours post oral administration, the plasma levels of the CBD Capsule group was over 2-fold higher than the naked CBD oil group, whilst in the brain, the CBD concentrations of CBD capsule and naked oil groups were low, but comparable. When CBD capsules were given in combination with DCA capsules (CBD capsule + DCA capsule group), the mean brain concentration of CBD was 1048 ng/mg tissue, a remarkable 40- and 30-fold increase compared to the naked CBD oil and CBD capsule groups, respectively. The lower plasma CBD concentration was observed in CBD capsule + DCA capsule mice compared to the CBD capsule mice, possibly a consequence of enhanced tissue uptake. After 1 hour of oral administration, brain CBD concentration of CBD capsule + DCA capsule mice showed over a 60% reduction to 418 ng/mg, while CBD capsule group increased from 42 ng/mg to 385 ng/mg and naked CBD oil group increased from 28 ng/mg to 380 ng/mg. The latter groups showed increases of over 800% to reach peak concentrations, resulting in comparable brain CBD levels across all three treatment groups. The potential effect of DCA on the drastic reduction in brain CBD concentration is an interesting finding that requires further investigations. Research suggests that bile acids my promote cellular uptake and clearance of lipoproteins [16], however the effects of bile acids such as DCA on tissue clearance of lipophilic drugs such as CBD have yet to be defined.

In plasma, the CBD capsule group mice showed a reduction of CBD from 0.3 hours, whilst a moderate decrease was observed in the naked group, reaching similar plasma CBD concentrations. The plasma CBD concentration of CBD capsule + DCA capsule mice showed only a slight decrease and remained substantially higher than the CBD capsule or naked CBD oil group mice. In these CBD capsule + DCA capsule mice, plasma CBD continued to reduce toward the 3 hour time point, whereas the CBD capsule and naked CBD oil groups showed no decline or only a slight decline, resulting in similar plasma CBD levels across all three groups at the 3 hour time point. The brain concentrations of CBD in CBD capsule, CBD capsule + DCA capsule and naked CBD oil mice showed only a modest reduction from the 1 hour to 3 hour time points, indicating comparable brain CBD concentrations.

The AUC graphs on the right show the cumulative abundance over 3 hours post-oral administration. The plasma concentrations of CBD were comparable between the CBD capsule group and naked CBD oil group. The 3 hour cumulative plasma CBD was substantially higher in CBD capsule + DCA capsule mice, showing more than double the concentration of CBD capsule, or naked CBD oil group (Fig 1B). Similarly, in the brain, the 3 hour cumulative CBD concentration was markedly higher and was double in the CBD capsule + DCA capsule mice compared to the CBD capsule mice (Fig 1D).

The trends described did not reach statistical significance using ANOVA (plasma: $p = 0.07$ and brain: $p = 0.67$), however, the treatment effect may be obscured by the small sample sizes. Indeed, our previous work reported significantly increased absorption of a lipophilic drug with similarly produced MVSA microcapsules in a mouse study with n = 10 per treatment group [11].

The PK parameters generated using PKSolver in Microsoft Excel are summarised in Table 1. Plasma terminal half-life ($t_{1/2}$) of CBD in mice that were given naked CBD oil was

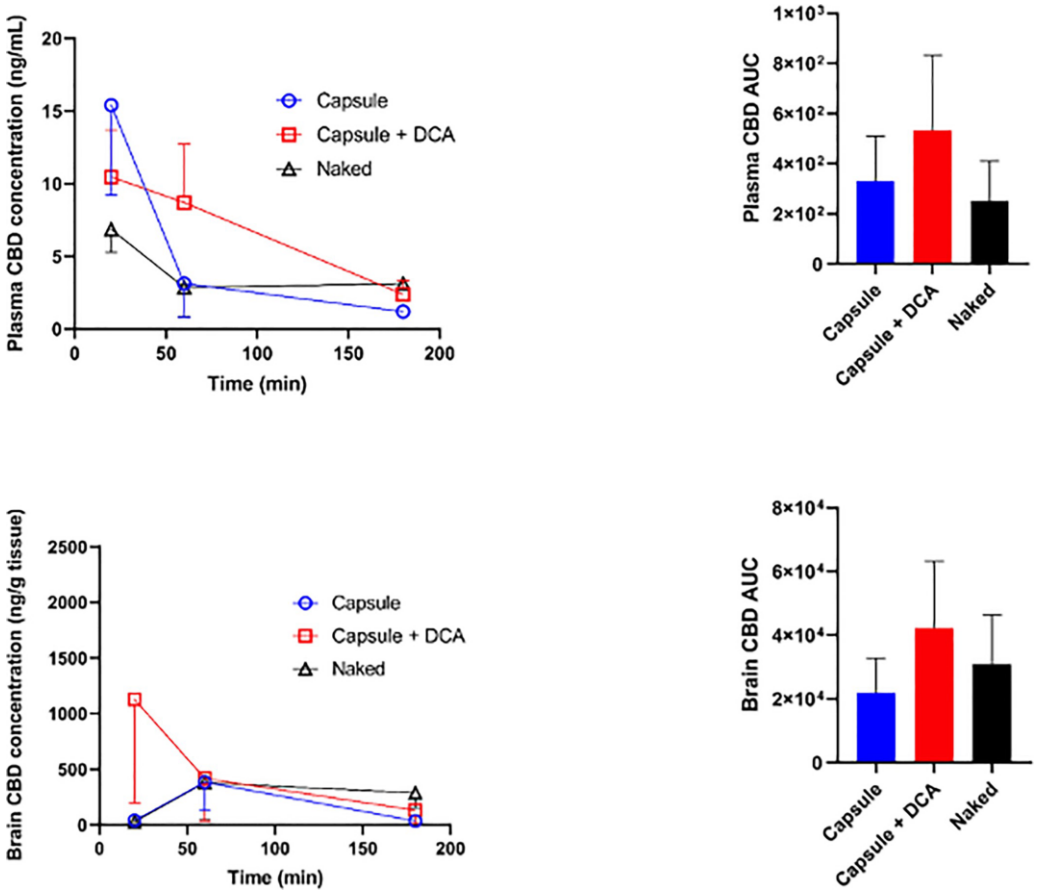

Fig 1. Mean concentration-time curve of Cannabidiol (CBD) in plasma and brain (a, c) with corresponding cumulative concentrations shown as area under curve (AUC) graphs (b, d). CBD was administered orally to mice at a dosage of 5 mg/kg according to the following formulations: 1) CBD capsules, 2) CBD capsules + deoxycholic acid (DCA) capsules (4 mg/kg) and 3) naked CBD oil. For each graph, the data represents Mean ±SEM bars (n = 3 or 4 per treatment group for each time point). Analysis using one-way ANOVA revealed no statistical significance, with plasma and brain data yielding $p = 0.07$ and $p = 0.67$, respectively.

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2.2 h. The plasma CBD concentration in the naked CBD group mice reached its peak at 0.3 h post-oral administration, which was 7.7 ng/mL. The plasma absorption profile of CBD oil was comparable with published findings [25], albeit with minimal variation in the pharmacokinetic parameters that are likely to be dose dependent [8]. The current results demonstrate that plasma bioavailability as indicated by C_{max} and $AUC_{0\text{-}t}$ was mildly increased for encapsulated CBD compared to naked oil (by 27.3% and 14.6%, respectively). With the same t_{max}, we conclude that capsulation may have a similar rate of intestinal absorption as naked oil, however, it may protect against extensive first-pass metabolism to promote CBD into the surrounding capillaries.

Table 1. Pharmacokinetic parameters of Cannabidiol (CBD) administered orally to mice at a dosage of 5 mg/kg in the following formulations: 1) capsule, 2) CBD capsule + deoxycholic acid (DCA) capsule (4 mg/kg) and 3) naked CBD oil formulation, in plasma and brain.

		Plasma			Brain		
Parameter	Unit	Capsule	Capsule + DCA	Naked	Capsule	Capsule + DCA	Naked
t1/2	h	1.1	0.4	2.2	0.9	4.5	Not determined
t_{max}	h	0.3	1.0	0.3	1.0	0.7	2.0
C_{max}	ng/mL or ng/g	10.9	11.0	7.7	280.9	1048.2	421.5
AUC 0-t	ng/mL*h	15.2	16.7	12.9	368.1	1242.1	447.4
AUC 0-inf_obs	ng/mL*h	16.3	6.5	14.9	75.0	4378.4	Not determined

Parameters were generated using standard non-compartmental analysis in PKSolver with plasma and brain concentration data.

Each value is expressed as Median.

$t_{1/2}$, terminal half-life; C_{max}, maximum concentration; t_{max}, time to reach C_{max}; AUC0–t, area under the concentration curve from zero to 3 hours post oral administration; AUC $_{0\text{-inf\_obs}}$, AUC from zero to observed infinity.

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The plasma $t_{1/2}$ of CBD was halved for the CBD capsule group mice compared to naked CBD group mice, which alludes to increased clearance via excretion and/or tissue/cell uptake. The plasma $t_{1/2}$ was shorter in CBD capsule + DCA capsule mice, indicating substantially enhanced CBD clearance from the plasma by the MVSA capsulation with DCA. Consistent with the latter, CBD analyses in brain tissue revealed in mice that were given CBD capsules, the t_{max} was 1 h, half of naked CBD group mice, indicating markedly faster brain tissue uptake of CBD by the MVSA capsulation. Furthermore, when the CBD capsules were administered with DCA in CBD capsule + DCA capsule group, the t_{max} was 1/3 of naked group mice, which also resulted in substantially higher C_{max}, AUC 0-t and AUC $_{0\text{-inf\_obs}}$. These data indicate markedly increased brain tissue uptake of CBD by the MVSA encapsulation in combination with DCA. Moreover, the mice that were administered with CBD or CBD capsule + DCA capsule showed substantially longer t1/2, indicating prolonged retention of CBD within the brain.

The limitations of the present study include the low number of mice for each treatment group, which may have affected the statistical analysis and obscured potential treatment effects. Additionally, the study would have been strengthened by increasing the number of time-points for sample collections. Subsequent investigations could include 5 timepoints to clarify the short-term blood-to-brain kinetics of CBD. With further optimisation of the CBD encapsulation, along with *in vitro* characterisation of CBD capsules, future studies are likely to increase the drug dosage so results are more comparable with published data.

Conclusion

Taken together, the outcomes of this study indicate that MVSA encapsulation may protect CBD from oxidation, degradation by light, and acidic digestion within the stomach, enhancing the absorption through the GI tract and cumulative plasma bioavailability. Although it was statistically non-significant, a bile acid, DCA, show increasing trend in uptake of CBD by up to 40 times within the brain and extends its retention. DCA may have a potential to promote the neuroprotective efficacy of orally administered CBD, particularly for the treatment of neurodegenerative disorders.

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APPENDIX 2: CO-AUTHOR ATTRIBUTION TABLES

Article 1: In vivo retinal imaging is associated with cognitive decline, blood-brain barrier disruption and neuroinflammation in type 2 diabetic mice.

	Conceptualisation and study design	**Experimental procedures**	**Data acquisition & statistics**	**Results interpretation**	**Manuscript writing**	**Critical review**
Co-Author 1: Maimuna Majimbi	65%	70%	70%	70%	100%	40%
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Article 2: S-allyl Cysteine and Cannabidiol are Equally Effective as Metformin in Preserving Neurovascular Integrity, Retinal Structure, and Cognitive Function in db/db Type 2 Diabetic Mice.

	Conceptualisation and study design	**Experimental procedures**	**Data acquisition & statistics**	**Results interpretation**	**Manuscript writing**	**Critical review**
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Article 3: Sodium alginate microencapsulation improves the short-term oral bioavailability of cannabidiol when administered with deoxycholic acid.

	Conceptualisation and study design	**Experimental procedures**	**Data acquisition & statistics**	**Results interpretation**	**Manuscript writing**	**Critical review**
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