Using the Pupillary Light Response to Measure Cerebrovascular Reactivity for Dementia Risk Assessment

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To my parents, who never doubted that I'd become Dr. Sparks someday.

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Research Dissemination

This section outlines research contributions made as the primary author, contributing to this thesis.

Peer-Reviewed Publications

The following chapters of this thesis contain material that has been disseminated in the following manuscripts, either published or in preparation:

Chapter 2

1. **S Sparks**, J Pinto, G Hayes, M Spitschan, D Bulte (2023). The impact of Alzheimer's disease risk factors on the pupillary light response. *Frontiers in Neuroscience (17)*. https://doi.org/10.3389/fnins.2023.1248640

Chapter 4

1. **S Sparks***, G Hayes*, J Pinto, D Bulte (2024). Characterising cerebrovascular reactivity and the pupillary light response – a comparative study. *Frontiers in Neuroscience (15)*. https://doi.org/10.3389/fphys.2024.1384113

   **: These authors have contributed equally to this work and share first authorship*

Chapter 5

1. **S Sparks**, G Hayes, D Bulte, J Pinto. Investigating the pupillary light response in the postpartum period. *Manuscript in preparation*.

Chapter 6

1. **S Sparks**, G Hayes, D Bulte, J Pinto. The sleep-mediated relationship between the pupillary light response and cerebrovascular reactivity. *Manuscript in preparation*.

Public Code Repositories

1. **cvd_pupillometry**: A fork of an existing repository to calculate pupillary light response characteristics, using the PupilLabs and Light Engine hardware and software along with the PyPlr Python library. *Open source (MIT License) at: github.com/BulteGroup/cvd_pupillometry*
2. **EyeBrainPupillometry**: A repository to calculate pupillary light response characteristics from NeurOptics using MATLAB and Python, as well as for statistical analysis of PyPlr data and NeurOptics PLR-3000 data for The Eye Brain Study. *Open source (MIT License) at: github.com/BulteGroup/EyeBrainPupillometry*
3. **MaternalBrainPupillometry**: A repository to calculate pupillary light response characteristics from the NeurOptics PLR-3000 using Python, as well as for statistical analysis of NeurOptics PLR-3000 data for The Maternal Brain Project. *Open source (MIT License) at: github.com/sierrasparks/MaternalBrainPupillometry*

Conferences

1. **S Sparks,** D Bulte, G Hayes, J Pinto (2025). “Investigating the pupillary light response in the postpartum period”. *ARVO Annual Meeting 2025*. Accepted abstract for poster presentation in May 2025.
2. **S Sparks,** J Pinto, G Hayes, D Bulte (2025). “The sleep-mediated relationship between the pupillary light response and cerebrovascular reactivity”. *AVA Annual Meeting 2025*. Accepted abstract for poster presentation in April 2025.
3. **S Sparks,** G Hayes, J Pinto, D Bulte (2024). “Evaluating the relationship between a standard model of VO2 max and end tidal CO2”. *The Podium Institute Inaugural Conference*. Published abstract. Poster presentation and flash talk.
4. **S Sparks** (2024)**.** The nine month stress test: is pregnancy a risk factor for dementia? *Alzheimer’s Research UK Thames Valley Network Research Day*. Research talk.
5. **S Sparks,** G Hayes, J Pinto, J Martin, M Spitschan, D Bulte (2024). “Comparing the pupillary light response using two pupillometers with cerebrovascular reactivity”. *Oxford Ophthalmological Congress 2024*. Published abstract. Poster presentation.
6. **S Sparks,** G Hayes, J Pinto, J Martin, M Spitschan, D P Bulte (2024). Comparing the pupillary light response to white, red, and blue stimuli with cerebrovascular reactivity. *ARVO Annual Meeting 2024.* Published abstract. Poster presentation.

7. **S Sparks**, D P Bulte, J Pinto (2023). Comparing brain amyloid load using PET to grey matter perfusion using ASL on the OASIS-3 dataset. *ISMRM Annual Meeting 2023*. Published abstract. Digital poster presentation.

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Abstract

With an ageing global population, the number of people living with Alzheimer's disease, the leading cause of dementia, is increasing and is expected to continue to increase over the next few decades, with no known cure for the disease. Because of this, there is an urgent requirement to determine a way to identify those at an increased risk for developing the disease, to enable targeted interventions.

Reduced cerebrovascular reactivity (CVR) has been reported in cases of neurodegeneration, which could be due to a smooth muscle disorder. This disorder could impact the muscles that control the contraction and dilation of the pupil and could potentially be observed through an impaired pupillary light response (PLR), and it is believed that this impairment could be observed before symptoms of cognitive decline occur. The aim of this research is to investigate the pupillary light response as a method for assessing cerebrovascular reactivity, and to use this information in conjunction with genetic, demographic, lifestyle, and life-event data to generate a risk classification for Alzheimer's disease, which considers the many potential root causes for the disease.

This thesis presents the first studies investigating the relationship between the PLR and CVR in healthy adults, as well as the first study to assess the PLR-CVR relationship with an integration of covariate factors relating to demographics and lifestyle factors. It also presents a comprehensive, peer-reviewed review of literature demonstrating how modifiable risk factors for Alzheimer's disease relate to the PLR, which can inform future risk classification models using the PLR and lifestyle information to assess dementia risk. Finally, this thesis presents the first study

evaluating differences in dynamic aspects of the pupillary light response in postpartum women and women who have never been pregnant, highlighting the need to consider sex-specific factors when assessing the PLR and subsequent dementia risk.

With more validation conducted in larger cohort studies, the work presented in this thesis supports the PLR as a potential screening tool for estimating cerebrovascular reactivity, and potentially subsequent dementia risk screening.

Abbreviations and Definitions

ACV	Average Constriction Velocity
AD	Alzheimer's Disease
ADAD	Autosomal Dominant Alzheimer's Disease
ANCOVA	Analysis of Covariance
ANOVA	Analysis of Variance
APOE	Apolipoprotein E
BMI	Body Mass Index
BOLD	Blood Oxygenation Level Dependent
BP	Blood Pressure
CBF	Cerebral Blood Flow
CVR	Cerebrovascular Reactivity
fMRI	Functional Magnetic Resonance Imaging
GWAS	Genome-Wide Association Study
ICP	Intracranial Pressure
ipRGC	Intrinsically Photosensitive Retinal Ganglion Cell
ISI	Interstimulus Interval
KSS	Karolinska Sleepiness Scale
MCA	Maximum Constriction Acceleration (Chapter 2 only)
MCA	Middle Cerebral Artery (Chapters 4 and 6)
MCV	Maximum Constriction Velocity
MDD	Major Depressive Disorder
MoCA	Montreal Cognitive Assessment

MRI	Magnetic Resonance Imaging
$PaCO_2$	Arterial Partial Pressure of Carbon Dioxide
PCA	Pupil Constriction Amplitude
$P_{ET}CO_2$	Percent End-Tidal Carbon Dioxide
PIPR	Post-Illumination Pupil Response
PLR	Pupillary Light Response
TBI	Traumatic Brain Injury
TCD	Transcranial Doppler Ultrasound
VSMC	Vascular Smooth Muscle Cell

Chapter 1: Introduction to Thesis

This thesis aims to investigate the pupillary light response as a method for assessing cerebrovascular reactivity, to aid in risk classification for dementia. This chapter includes an overview of the motivation, context, and significance of the research included in this thesis, including background information about Alzheimer's disease and dementia, cerebrovascular reactivity, and the pupillary light response. Further, it presents the goals and main research questions that this thesis aims to address, within the defined scope of the thesis. Finally, this chapter outlines the content of each research chapter included in this thesis.

1.1. Study Context and Motivation

With an ageing global population, the number of people living with dementia is increasing, and is projected to continue to increase over the next few decades, especially in those living in low- and middle-income countries [1]. Importantly, Alzheimer's disease (AD), which is the most common cause of dementia [1], is believed to occur at least 20 years before symptoms of the disease arise [2]. It takes years of changes occurring in the brain before individuals with AD experience noticeable symptoms including memory loss and language problems [2]. As such, there is an urgent requirement to determine a way to prevent the disease and to delay the onset of symptoms impacting daily livelihood. To prevent Alzheimer's disease, it is important to understand steps in the disease's pathogenesis, including an investigation into genetic, lifestyle, and life event factors that contribute to the disease risk and severity, and to investigate tools that could be used in its pre-symptomatic phase to allow for interventions [3]. Critically, incorporating an analysis

of genetic, lifestyle, and environmental risk factors, which may all contribute to AD risk [4], [5], [6], is important for addressing the many root causes of AD and dementia.

Studies have shown that with increasing age, there is a dysregulation of cerebral blood flow (CBF) in response to neuronal and metabolic demands, due to impaired signalling between elements of the neurovascular unit [7]. Cerebrovascular reactivity (CVR) is a measure of the response of blood vessels in the brain to changes in demand and to external stimuli, and impaired CVR has been indicated in a wide variety of neurological disorders including dementia, multiple sclerosis, and stroke [7] [8] [9] [10] [11]. Additionally, it has been shown that in many cases, impaired CVR is demonstrable several years before an official dementia or Alzheimer's disease diagnosis [8]. Since Alzheimer's disease is thought to begin 15 to 20 years before an official diagnosis [9], early detection through the assessment of CVR may be a valuable tool.

Many methods can be used to measure CVR, however a common issue is their invasiveness or low spatial resolution [7]. As an alternative, magnetic resonance imaging (MRI) has advantages in that it can offer richer spatial information which aids with CVR mapping, while maintaining a minimal level of invasiveness to the patient [10]. MRI currently seems to be one of the best imaging modalities for CVR assessment, especially when prioritizing patient comfort and safety [11].

However, MRI alone is not suitable for large-scale data collection for CVR assessment. MRI machines are not only physically large, but they are expensive and lack widespread availability, meaning that it can take a significant amount of time

just to schedule a scan. As such, an alternative solution is necessary. Transcranial Doppler ultrasound (TCD), which can assess blood flow velocity in cerebral arteries, can also be used to assess CVR [12], [13], at a much lower cost and thus with higher accessibility in various settings.

A potential alternative is to investigate one of the many root causes of impaired CVR leading to an increased risk of dementia, one of which could be due to a smooth muscle disorder, potentially observable in the smooth muscle that controls the constriction and dilation of the pupil. Pupillary size is controlled not only by the smooth muscle but by the opposing branches of the autonomic nervous system [14], and dysfunction in this muscle could manifest in an impaired pupillary response to light and could indicate implications to the parasympathetic and sympathetic nervous systems. It is hypothesized that this impairment may occur before an official AD diagnosis is made, which means that this could be used for diagnostic purposes before other symptoms, specifically cognitive deficits, arise.

Further, it is hypothesized that the mechanisms that account for CVR impairment will determine whether the pupillary light response (PLR) is impacted. CVR impairments that are intrinsic, potentially due to a smooth muscle disorder, may manifest as an impaired PLR, in those with genetic or otherwise unknown risk factors for AD. Extrinsic CVR impairments, due to lifestyle or environmental factors, may have a different impact on the PLR which is distinct from the intrinsic CVR impairments. To make these distinctions, the combination of the PLR assessment and physiological, lifestyle, demographic, and life-event data is required.

Impaired PLR has been shown in current dementia cases, but it has not been investigated as a risk-prediction tool in at-risk populations. Given both the PLR and CVR's relation to smooth muscle function and dementia, in addition to several other pathologies, there is a requirement for further investigation into the relationship between the PLR and CVR. This could be used to determine whether the PLR can act as a screening tool, in conjunction with lifestyle and genetic factors, to provide an estimate of CVR and subsequent risk for dementia.

1.2. Thesis Aim and Scope

The objective of the proposed research is to investigate the pupillary light response as a method for assessing cerebrovascular reactivity, and to use this information in conjunction with genetic, demographic, lifestyle, and life-event data to generate a risk classification for Alzheimer's disease, which considers the many potential root causes for the disease. This could then be used for targeted interventions based on an individual's risk profile, which may differ due to specific risk factors present.

This thesis aims to answer the following research questions:

1. How do established Alzheimer's disease risk factors influence the pupillary light response?
2. What parameters of the pupillary light response relate most strongly to cerebrovascular reactivity?
3. How do genetic, lifestyle, environmental, and demographic factors influence the relationship between the pupillary light response and cerebrovascular reactivity?

These specific research questions aim to contribute to the overall objective of assessing the PLR as a method for assessing CVR, in conjunction with other covariates relating to dementia risk, to assess an individual's risk for dementia.

This thesis addresses the above questions in a department-approved integrated thesis. This thesis contains two scholarly articles fully published in peer-reviewed, major journals (*Frontiers in Neuroscience, Frontiers in Physiology*) [15], [16], as well as manuscripts in preparation for submission and work accepted and presented at peer-reviewed national and international conferences. As research in engineering is collaborative in nature, I have included recognition of individuals who have contributed to work included in each research chapter, where relevant.

This research was conducted between October 2021 and February 2025 at the University of Oxford, UK, based at the Oxford Institute of Biomedical Engineering and the Oxford Centre for Human Brain Activity. All methods were developed in-house, and data was collected with volunteers in-person in Oxford, UK, after acquiring ethics approvals from the University of Oxford for specific studies.

This research was conducted, in-part, during the COVID-19 pandemic, which caused significant disruption into the ethics approval process as well as the ability to conduct research with volunteers in-person. As such, the sample sizes for the studies included in this thesis are limited, as the studies could not be conducted until it was safe to do so for both the volunteers and researchers. Additionally, as the imaging resources available to the researchers were based in Oxford, all study participants had to either be based in Oxford or be willing to travel to Oxford to participate in the study, which meant that the study cohorts were biased to the local

university populations and the cohorts do not necessarily represent what would be expected in the UK at large, or globally. Despite these limitations in the study cohorts, this thesis provides valuable insights into methods for assessing the PLR and in comparing this to CVR and other factors impacting dementia risk, which support the need for future work in this area, particularly with larger, more diverse cohorts based in other locations.

As this research aimed to investigate the PLR as a potential risk screening tool rather than a diagnostic tool for those with dementia or other neurodegenerative diseases, none of the study participants had any diagnosed form of neurodegeneration and were all classified as cognitively healthy at the time of data acquisition using the Montreal Cognitive Assessment (MoCA) [17]. As the data for this thesis was collected throughout the period of study toward the DPhil, it was not within the scope of this research to conduct a longitudinal study that follows-up on the cognitive status of study participants to determine whether they do develop dementia in the decades to come. Instead, within the scope of this research, participants with elevated genetic and lifestyle risks for dementia were included as well as those classified as low risk, and the measurement of cerebrovascular reactivity was used to estimate comprehensive risk for dementia.

To address the research questions and overall objective, this thesis includes an investigation into the impact of Alzheimer's disease risk factors on the PLR, to account for any confounding factors or comorbidities between risk factors and the PLR that must be accounted for. Volunteers have been recruited for two studies

based at the University of Oxford: The Eye-Brain Study (Chapters 3-4, 6), and The Maternal Brain Study (Chapter 5).

The Eye-Brain Study include a genetics assessment (to determine APOE status – an important genetic allele with significant links to Alzheimer's disease risk), demographics and lifestyle assessment (to assess demographics and risk factors such as age, sex, medical and pregnancy history, physical activity levels, and level of education, among others), basic physiological measurements, and an assessment of their PLR. The PLR is being assessed with various light protocols to allow comparisons between rod, cone, and melanopsin-driven responses, as well as the parasympathetic and sympathetic nervous systems, to determine whether there are impacts with specific photoreceptor or nervous systems. To estimate CVR, data from transcranial Doppler ultrasound with carbon dioxide gas challenges is being used to get an estimate of CVR from blood flow velocity and changes in oxygen and carbon dioxide concentrations. To confirm CVR values, data from functional MRI scans will be used.

The Maternal Brain Study similarly includes a genetics assessment, demographics assessment, PLR and CVR assessment, and a more extensive assessment of factors relating to women's health to identify the impact of women's health factors, such as pregnancy, on the PLR and CVR.

1.3. Research Significance

The work presented in this thesis has contributed original research to the fields of biomedical engineering, physiology, neuroscience and vision sciences. Importantly, it also has the potential for future significant contributions to research in biomedical

engineering and medicine as the methods presented in this thesis are still in-use in the Eye-Brain Study and the Maternal Brain Study.

This thesis presents the first studies investigating the relationship between the PLR and CVR in healthy adults, as well as the first study to assess the PLR-CVR relationship with an integration of covariate factors relating to demographics and lifestyle factors. It also presents a comprehensive, peer-reviewed review of literature demonstrating how modifiable risk factors for Alzheimer's disease relate to the PLR, which can inform future risk classification models using the PLR and lifestyle information to assess dementia risk. Finally, this thesis presents the first study evaluating differences in dynamic aspects of the pupillary light response in postpartum women and women who have never been pregnant, highlighting the need to consider sex-specific factors when assessing the PLR and subsequent dementia risk.

With more validation conducted in larger cohort studies, the work presented in this thesis supports the PLR as a potential screening tool for estimating cerebrovascular reactivity. If successful, the PLR could be an inexpensive, fast, and accessible tool for cerebrovascular reactivity assessment and potentially subsequent dementia risk screening. This would help healthcare professionals with identifying patients with a higher dementia risk, without the need for an expensive or time-consuming MRI scan, and devices that assess the PLR could be used in routine medical check-ups for early screening.

Building on the research presented in this thesis, a risk classification model could be created to be used for targeted interventions based on an individual's risk profile

incorporating the PLR and lifestyle, genetic, demographic and environmental factors for dementia risk prediction, enabling more individualised intervention.

1.4. Outline of Thesis

To evaluate the research questions and to meet the objective presented in Section 1.2, this thesis includes a literature review, pupillometry tools and methods development, and pilot studies.

Chapter 2 presents a comprehensive, peer-reviewed literature review outlining key risk factors for Alzheimer's disease and their relation to the PLR, if any (addressing research question 1) [15]. This starts with an introduction to Alzheimer's disease and current research investigating Alzheimer's disease and the eye, including the retina, eye movements, and the study of the pupil in current AD patients. It then introduces the field of pupillometry and the pupillary light response and identifies key knowledge gaps in the field of pupillometry for AD risk assessment. The review focuses on 12 potentially modifiable AD risk factors relating to lifestyle and environmental factors, and how they relate to the pupillary light response. It also summarises various pupillary stimuli and measurements, as well as the physiological and autonomic nervous system pathways contributing to the PLR.

Using the reviewed literature, **Chapter 3** presents an overview and assessment of various parameters and protocols for assessing the PLR and post-illumination pupil response (PIPR) to determine the best methods and tools for subsequent research presented in this thesis (contributing to research question 2). Importantly, it compares the pupillometry tools available within the scope of this research – a NeurOptics PLR-3000 handheld pupillometer, and an in-house system of hardware

and software enabling the use of binocular assessment of chromatic pupillometry [18].

Chapter 4 builds on the findings of the best methods and tools available within the research scope and presents a first application of these methods in a pilot study. This study compares the PLR to a positive and negative white light stimulus with steady-state CVR in healthy adults, to determine a standard relationship between parameters of the PLR and CVR (addressing research question 2) [16].

As part of the desire to implement inclusive science into this thesis, it was important to not only consider the PLR in isolation but to consider how other, understudied factors may influence the PLR and subsequent PLR-CVR relationship, including some sex-specific factors. **Chapter 5** addresses this by using pilot data from the Maternal Brain Study to assess the PLR in postpartum women, ultimately to determine whether a history of pregnancy impacts the PLR (contributing to research questions 1 and 3).

Chapter 6 returns to data collected in the Eye-Brain Study to assess both the PLR and PIPR with linear CVR in a more diverse cohort. This cohort includes those considered low risk for dementia, and those with established lifestyle and genetic risk factors for dementia. A covariate analysis was done to evaluate the impact of specific factors including age, sex, and sleep quality on the PLR/PIPR-CVR relationship (addressing research questions 2 and 3).

Finally, this thesis concludes with **Chapter 7**, where a summary of the main findings of each chapter and answers to the main research questions are presented. The

overall contributions and significance of this research are highlighted, along with limitations of this thesis and potential areas for future research.

Chapter 2: The impact of Alzheimer's disease risk factors on the pupillary light response

The aims of this chapter are to review the most prevalent lifestyle risk factors to Alzheimer's disease, discuss the pupillary light response, and investigate the comorbidity of lifestyle and genetic risk factors for Alzheimer's disease and to see how they relate to the pupillary light response. Although the impaired pupillary light response has been studied in current Alzheimer's disease subjects, this has not been extensively investigated in at-risk groups for the disease. Determining whether the pupillary light response is similarly impaired in specific at-risk subject groups before an Alzheimer's disease diagnosis is made is an important step before using the pupillary light response as a diagnostic or screening tool.

The work from this review can be used to identify key at-risk groups where an impaired pupillary light response may be indicative of increased Alzheimer's risk and could identify groups where preventative measures should be taken before onset of the disease. Understanding and analysing the extent to which the pupillary light response is impaired given certain lifestyle conditions will be essential to assessing Alzheimer's disease risk based on the pupillary light response and a lifestyle, physiological, and genetic analysis, which is the aim of future chapters.

This chapter relates to Research Question 1 and was adapted from the publication:

S Sparks, J Pinto, G Hayes, M Spitschan, DP Bulte, DP (2023). "The impact of Alzheimer's disease risk factors on the pupillary light response." *Frontiers in Neuroscience (17)*. https://doi.org/10.3389/fnins.2023.1248640

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2.1. Abstract

Alzheimer's disease (AD) is the leading cause of dementia, and its prevalence is increasing and is expected to continue to increase over the next few decades. Because of this, there is an urgent requirement to determine a way to diagnose the disease, and to target interventions to delay and ideally stop the onset of symptoms, specifically those impacting cognition and daily livelihood. The pupillary light response (PLR) is controlled by the sympathetic and parasympathetic branches of the autonomic nervous system, and impairments to the pupillary light response (PLR) have been related to AD. However, most of these studies that assess the PLR occur in patients who have already been diagnosed with AD, rather than those who are at a higher risk for the disease but without a diagnosis. Determining whether the PLR is similarly impaired in subjects before an AD diagnosis is made and before cognitive symptoms of the disease begin, is an important step before using the PLR as a diagnostic tool. Specifically, identifying whether the PLR is impaired in specific at-risk groups, considering both genetic and non-genetic risk factors, is imperative. It is possible that the PLR may be impaired in association with some risk factors but not others, potentially indicating different pathways to neurodegeneration that could be distinguished using PLR. In this work, we review the most common genetic and

lifestyle-based risk factors for AD and identify established relationships between these risk factors and the PLR. The evidence here shows that many AD risk factors, including traumatic brain injury, ocular and intracranial hypertension, alcohol consumption, depression, and diabetes, are directly related to changes in the PLR. Other risk factors currently lack sufficient literature to make any conclusions relating directly to the PLR but have shown links to impairments in the parasympathetic nervous system; further research should be conducted in these risk factors and their relation to the PLR.

2.2. Introduction

2.2.1. Alzheimer's disease

With an ageing global population, the number of people living with dementia is increasing, and is projected to continue to increase over the next few decades, especially in those living in low- and middle-income countries [19].

Importantly, Alzheimer's disease (AD), which is the most common cause of dementia [19], is believed to occur at least 20 years before symptoms of the disease arise [20]. It takes years of changes occurring in the brain before individuals with AD experience noticeable symptoms including memory loss and language problems [20]. As such, preventing Alzheimer's depends primarily on understanding early steps in the disease's pathogenesis, including an investigation into genetic factors and potential biomarkers that could be identified in its pre-symptomatic phase [21].

2.2.2. Alzheimer's disease and the eye

Along with symptoms that affect cognition, patients who have been diagnosed with AD often display other biological characteristics, which can, to varying extents, be used in disease monitoring and potentially in diagnosis. One category of biological characteristics includes changes to the eye that occur in the early stages and during the progression of the disease, which will be explored further in this section.

2.2.2.1. State of the art

Research into the eye and its relation to cognitive decline, including in both preclinical and onset AD, has been the focus of several recent studies and reviews. Many such reviews investigate changes and degeneration in the retina, and how this can relate to neurodegeneration [22], [23], [24], [25], [26], [27], [28], [29]. These reviews discuss the role of amyloid-beta (Aβ), a biomarker for AD, in the retina and in patients with glaucoma [22], [23], [26], [27], [28], and confounding factors in the eye such as retinal nerve fibre layer thickness thinning that can be observed in some cases of neurodegeneration [23], [24], [28], [29]. Other studies involving the retina also discuss accumulation of phosphorylated tau in the brain, another biomarker of AD, and how this often relates to an accumulation of tauopathy in the retina in cases of AD [23], [27], [30], [31]. In AD, tau-related changes cause retinal neuron dysfunction and subsequent death, which contributes to visual deficits in AD [30]. In cases of AD, tau related changes in the retina may be more consistent than amyloid beta changes in the retina, suggesting that phosphorylated tau in the retina may be a promising biomarker for AD [32].

Despite its prevalence in current research, analysing changes in the retina has established limitations as a diagnostic tool for AD and dementia because of the comorbidity with AD risk factors such as hypertension, diabetes, and retinopathy [25], [33], [34]. As such, it can be difficult to differentiate damage done to the retina from these diseases from damage done due to potential neurodegeneration. Additionally, tests such as measuring the retinal nerve fibre thickness can lack sufficient specificity and sensitivity for broader clinical applications [27].

Some studies have suggested that tracking eye movement abnormalities is an indicator of cognitive decline that can be used as a diagnostic tool for assessing the progression of AD [35], [36]. Others have analysed the effects of tropicamide on the pupil dilation response, showing that the pupil dilation is altered in Alzheimer's patients compared to healthy people [37]; however, there have been other studies that have not shown this to be consistently statistically significant and so has limitations as a diagnostic aid [38].

Further studies involving pupillometry applied to cognitive decline include identifying changes in the velocity and acceleration of pupil constriction in those with cognitive deficits [39]. Pupillary changes have also been used to assess subject response to varying cognitive loads and the ability for those with and without cognitive impairment to adapt cognitive effort [40].

2.2.2.2. Pupillometry

The study of pupillometry has been around for many years. Granholm claims that changes in pupillary motility have been observed and used as indicators of medical state and emotional arousal for over two millennia [41]. Loewenfeld cites Fontana's

work in 1765 as the earliest documentation of what was then known as “paradoxical pupil dilation”, or pupil dilation without changes in illumination [42]. The work by Lowenstein and Loewenfeld was essential to the field of pupillometry, and their influential work is summarized in their textbook from 1999, The Pupil, which has been a standard reference on the pupil [43].

Of note, the study of pupillometry has become increasingly more popular since the 1980s [44]. With this, there have been more studies that have looked at how various visual stimuli can be used to evoke a pupil response, and in turn what this response may be related to. For researchers in preventative medicine, pupillometry has been a valuable and inexpensive tool for screening for diseases such as diabetes and cardiac autonomic neuropathy [33], [45].

The activity of human photoreceptors can control pupil size, which has best been shown by studies examining pupil size using the method of silent substitution – where pairs of lights are alternated to only stimulate one photoreceptor class at a time [46]. These photoreceptors, consisting of melanopsin, rods, and cones, contribute to the control of the pupil in different ways, and in different temporal regimes [46]. Between 1 and 10 s from the onset of light exposure, cones and rods account for pupil constriction; melanopsin largely controls pupil size at 100 s, with some contribution from the rods [46], [47]. Further, rods are not expected to contribute to pupil control at photopic light levels due to rod saturation [46], [48], while cone receptors and melanopsin-containing intrinsically photosensitive retinal ganglion cells (ipRGCs) are active during daylight and contribute to the constriction of the pupil [49].

When assessing potential pupillary changes in neurodegeneration, it is important to consider how these various psychological and physiological aspects may impact a response, and to account for these in a potential protocol.

Pupillary Light Response

Outside of research assessing the retina's role in neurodegeneration and general eye-tracking, there are other potential biomarkers involving the eyes that are important in AD research. Pupillometry at large has been applied extensively in the study of cognition [50].

Of particular interest to this review are the studies that have investigated changes in the pupillary light response (PLR) in subjects with cognitive impairment, which assesses how the pupil dilates and constricts in different light conditions [33], [45]. Although some studies have failed to show a relationship between an impaired PLR and cognitive impairment [39], there have been several studies that have suggested that changes to the PLR may occur in patients with AD [51], and even in patients in the preclinical phase of AD [52]. These studies also suggest that dynamic pupillometry, and assessment of the PLR, could be useful tools in medical research to monitor the progression of cognitive decline, in addition to being used as a non-invasive, cost-effective screening tool for AD [51], [52], [53].

Most of the studies that assess the PLR occur in patients who have already been diagnosed with AD, rather than those who are at a higher risk for the disease due to genetic or non-genetic risk factors but who are not currently diagnosed with the disease. Because of this, it is difficult to determine whether the PLR is impaired because of the disease, or if an impaired PLR can indicate an elevated risk for later

development of the disease. It would, therefore, be useful to know if the PLR is similarly impaired in subjects before an AD diagnosis is made and before cognitive symptoms of the disease begin. Specifically, it would be helpful to know whether the PLR is impaired in specific at-risk groups, considering both genetic and non-genetic risk factors. It is possible that the PLR may be impaired in association with some risk factors but not others, potentially indicating different pathways to neurodegeneration that could be distinguished using PLR. If the PLR is impaired in individuals in specific risk groups for AD before symptoms of AD progress to a diagnosis, the PLR could then provide a quantitative measure to assist in predicting a person's risk for developing AD in conjunction with a genetic, life event, and lifestyle analysis, and thus be used to identify potential patient-specific early interventions.

This review aims to bridge the gap in current literature that focuses on the impairment in AD patients, and to expand this to include the analysis of the PLR in groups displaying individual risk factors for the disease before a diagnosis is made. The aim is to investigate how these risk factors relate to one another, in addition to analysing their relation to an altered PLR, if any, to determine whether the PLR is impaired in any at-risk groups for AD prior to diagnosing the disease. With the prevalence of pupillometry in current research, and the demand for a means of diagnosing preclinical AD with an inexpensive and accurate tool, analysing the PLR of subjects who may be susceptible to developing the disease later in life could be a valuable area of research which, if successful, could lead to further advancements in the prevention of AD.

2.2.3. Goals

The specific objectives of this review are to provide an overview of the most prevalent AD risk factors (genetic and non-genetic), discuss pupillometry and the PLR, and investigate the relationship between AD risk factors and PLR.

In particular, this review focuses on research that has been done relating the PLR to specific lifestyle factors that have been linked to AD risk.

2.3. Alzheimer's disease risk factors

There are many risk factors for AD and dementia, which can be split broadly into two categories: genetic risk factors, and non-genetic risk factors.

2.3.1. Genetic risk factors

Intrinsically, AD, specifically early onset AD, is often caused by mutations in one of three genes: amyloid precursor protein, presenilin 1, and presenilin 2 [54], [55]. Late onset AD is not necessarily as predictable but can be indicated by inheritance of the ε4 allele of the APOE gene [54], [56]. The inheritance of the APOE ε4 allele is the strongest genetic risk factor for AD – although only about 20-25% of the population carries one or more ε4 alleles, 50-65% of people diagnosed with AD carry the allele [55].

The inheritance of one or more ε4 alleles has implications on the age of onset of AD. Having at least one ε4 allele is associated with a reduced onset age for AD, and people with two ε4 alleles can develop AD up to 10 years earlier than those without the allele [55]. Despite being associated with the age of onset of AD, it is not clear whether carrying the ε4 allele is also a risk factor for a faster progression of the

disease once dementia has been reached. Contrary to the ε4 allele, the presence of the ε2 allele can help to reduce the risk of developing AD [55].

Using functional MRI, studies have shown that the ε4 allele moderates brain function [57]. This moderation of brain function includes changes in white matter integrity and brain connectivity, and may make the brain more susceptible to age-associated pathological mechanisms such as amyloid beta accumulation [58]. Further, this moderation is evident in young adults decades before any potential cognitive decline [57]. However, other functional MRI studies using blood-oxygenation-level-dependent (BOLD) contrast have reported similar BOLD activity in both ε4 and ε2 carriers, despite the expectation that the high-risk ε4 carriers would have an opposite activation to low risk ε2 carriers; it is thus necessary to consider more than the functional MRI signal to determine the relationship between APOE, AD risk, and brain function [57].

Genome-wide association studies (GWASs) are a powerful tool that have not only been responsible for the confirmation of APOE as a genetic risk factor for late-onset AD [59], but have aided in the discovery of other potential genetic candidates that may influence AD risk [60], [61], [62]. Interestingly, GWASs have also identified overlapping risk genes between AD and cardiovascular factors, including ischemic stroke [63] and plasma lipids [64], which motivates further research into vascular factors relating to AD and cerebral health.

2.3.2. Non-genetic risk factors

There are several extrinsic, life-event, or lifestyle-based, risk factors for AD that have been identified. Historically, dementia was not considered to be preventable or

treatable; within recent years progress has been made to identify non-genetic risk factors for the disease and to collect information on preventing and managing the disease [19]. Although the underlying symptoms and illnesses with dementia may not be curable, the current understanding is that the progression and handling of the disease can be manageable when considering these non-genetic, lifestyle-based risk factors and factors that are considered to be protective against the disease, including aspects of diet, physical activity, and levels of cognitive reserve [19], [65].

Dementia is most common among adults aged 65 years or older, which is incidentally when age-related physical health problems and dementia co-occurring is common [19]. Additionally, these physical health problems often overlap with the lifestyle-based risk factors that increase the risk of dementia; an impaired mental and physical function may interfere with a person's regular scheduling of things such as exercise and social interactions, all of which can further contribute to dementia risk [19]. Further, ethno-racial and socioeconomic factors can have an important impact on a person's lifestyle, and so these factors must be considered as well and research conducted into dementia risk factors within individual populations cannot be considered adequate to apply to all people [6], [66]. As such, when considering some lifestyle-based risk factors and their specific contributions to dementia risk, it is necessary to consider the comorbidity of individual genetic, lifestyle, social, cultural, and economic risk factors, in dementia cases.

In 2017, The Lancet Commission published an in-depth analysis of the main, potentially modifiable, risk factors for AD [19]; this list was updated in 2020 with three additional factors identified [6]. This analysis sought to estimate the Population

Attributable Factor (PAF), defined as the percentage reduction in new dementia cases over a given time if a specific risk factor were eliminated completely, for known modifiable risk factors for dementia [19]. The risk factors that were included in the PAF calculations were chosen by identifying risk factors listed in the UK National Institute of Health and Care Excellence (NICE) and US National Institute of Health (NIH) guidelines. Specifically, The Lancet Commission has identified 12 main categories for modifiable risk factors, through a systematic review and meta-analysis [6]. As will be shown throughout this review, these risk factors appear in a notable number of recent studies, and so these were taken to be the basis of this review's focus. These risk factors, and their relative weightings in terms of the percentage of AD cases they cause, are categorized into early life (age < 45 years), midlife (age 45-65 years), and late life (age > 65 years) [6]. These risk factors are shown in Table 2.1.

Table 2.1: *Potentially modifiable risk factors for AD and their PAF, as calculated by Livingston et al*. [6].

Category	Risk Factor	PAF
Early life, potentially modifiable	Less education	7%
Mid-life, potentially modifiable	Hearing loss	8%
	Traumatic Brain Injury	3%
	Hypertension	2%
	Alcohol consumption (greater than or equal to 14 units/week)	1%
	Obesity	1%
Later life, potentially modifiable	Smoking	5%
	Depression	4%
	Social isolation	4%
	Physical inactivity	2%
	Air pollution	2%
	Diabetes	1%
Risk unknown		60%

Although there are potentially other risk factors for dementia, these 12 main lifestyle/life event risk categories can be used to form a basis of factors to analyse

the comorbidity of risk factors and to assess their relation to other biomarkers of AD. It is important to acknowledge however, that the evidence that has been collected by Livingston *et al*. about these AD risk factors is from high income countries, and thus these risks could differ in other countries and corresponding interventions may require modifications in specific environments [6]. In this review, we will be assessing these 12 lifestyle and life event categories with their relation to the parasympathetic/sympathetic pathways and to the pupillary light response, if any.

2.4. Pupillometry and parasympathetic/sympathetic pathways

2.4.1. Pupillary stimuli and measurements

Pupillometry has been established as a promising means for assessing cognitive function, among other cerebral and bodily functions. Ultimately, the size and responsiveness of pupils in humans is controlled by the two main branches of the autonomic nervous system: the sympathetic and parasympathetic nervous systems, which control the dilator and sphincter muscles in the iris, respectively [67], [68]. Figure 2.1 shows the sphincter and dilator muscles on a constricted and dilated pupil. Further, pupillary constriction, accommodation, and vergence make up the near triad visual response, which enables a focused image, increased depth of focus, and binocular vision [69], [70]. To maximize the impact of using pupillometry to assess specific functions, namely parasympathetic and sympathetic functions, it is necessary to stimulate the pupil and measure concomitant pupil size changes with a measurement system that is best suited to measure these changes.

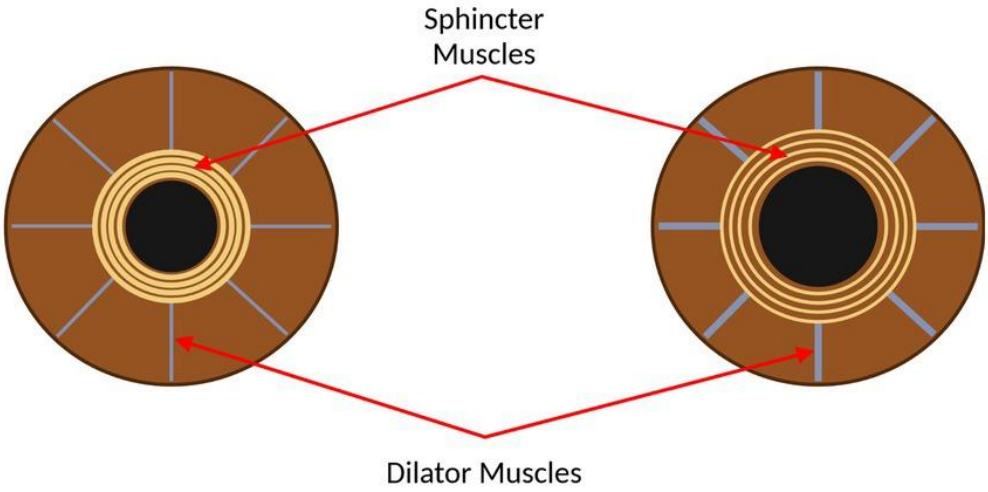

Figure 2.1: *The eye, with the sphincter and dilator muscles labelled. Left: The eye with the pupil constricted. Right: The eye with the pupil dilated. This figure was created by SS using BioRender.com.*

2.4.1.1. Pupillary measurement systems

One concern when using pupillometry as a diagnostic tool is ensuring consistency of measurement across different individuals with different eye sizes, and these individual differences must be accounted for. Usually, these differences are accounted for by expressing values relative to a baseline. Many pupillary measurement systems assess relative changes in either the pupil size or the ratios between the pupil and iris. One measurement method to correct for differences in eye sizes between subjects, as described by Fotiou *et al.*, is to use the ratios between the current pupil radius (P), the baseline pupil radius (B), and the iris radius (I) at each measurement point in time [51]. Fotiou *et al*. tested the use of the pupil-to-baseline pupil ratio (P/B) to determine which would be most suitable to produce the most reliable results, and the result of their study was that the pupil-to-iris method was preferred as the baseline pupil size is difficult to keep consistent even within the same individual, whereas the iris size is a stable anatomical marker [51]. The pupil-to-iris (P/I) ratio is a popular method that has been used by multiple researchers [51], [71], [72].

When making pupillary measurements, it is also important to identify an appropriate time and sampling frame for measurements. The pupil is constantly changing size due to small oscillations of the pupil, known as hippus [67]. Due to these continuous changes, it is not reliable to take a single measurement of the pupil size. For the most accurate measurements, the pupil size should be sampled frequently over a suitable time period to obtain reliable measurements that can account for these minor oscillations [67].

One way in which continuous measurements can be made is by using automated measurements through deep learning techniques. One web app that uses deep learning for translational and real-time pupillometry is MEYE, developed by Mazziotti *et al*. [73]. To make the pupillometry measurements, they applied random rotation, cropping, horizontal and vertical flipping of images, in addition to random brightness, contrast, and sharpness changes to train the model [73].

2.4.1.2. Pupillary stimuli

There are many ways to stimulate pupillary changes – each of which has different diagnostic purposes. Pupillometry studies vary widely in their stimuli, and without a standard methodology used across all pupillometry-related studies, there is a challenge presented in comparing the results of these studies [74]. Despite this, studies using similar methods can still be compared.

Many studies have shown how cognitive processes can cause pupillary changes – specifically, emotional arousal, interest, and task difficulty [75], [76], [77]. When the cognitive task demand is increased over time, the pupil dilates following this stimulus, and then constricts when the subject has less difficulty with the cognitive

task at hand [78], [79]. Further, when performance is sustained during a difficult task, this is modulated by the cortical inhibition of the parasympathetic pathway located at the oculomotor nucleus [79].

Another stimulant that has been used in pupillometry studies, specifically when assessing potential neurodegeneration, is a dilute solution of tropicamide. Administering tropicamide can block the parasympathetic sphincter muscle, which impacts the pupillary reaction [79]. Scinto *et al*. show that AD patients, or probable AD patients who have not yet been diagnosed with AD, have a more pronounced pupillary reaction and hypersensitivity to a dilute solution of tropicamide when compared to normal controls, suggesting that their parasympathetic sphincter muscle works abnormally when compared to normal controls [80]. Further, Higuchi *et al*. show that subjects with the APOE ε4 allele have a more hypersensitive response to tropicamide [81]. However, not all studies agree on the effects of tropicamide on pupillary reactions in AD patients – Granholm *et al*. found that AD patients did not differ significantly in pupillary responses to tropicamide when compared with cognitively normal controls [82]. Although Granholm *et al*. attempted to use similar methods to Scinto *et al*., including a 0.01% dilute solution of tropicamide, there may have been variability between the subject groups of the studies that could account for different results. Granholm *et al*. note that ethnicity, eye colour, age, and background luminance may be important factors that could impact the pupillary response to tropicamide, and so their study tested both light and dark conditions and had subjects similar in age, gender, eye colour, and ethnicity [82]; in contrast, Scinto *et al*. did not report ethnicity, eye colour, or background luminance in their methods, which could explain their conflicting results

with Granholm et al. [80]. Additionally, neither Scinto *et al*. nor Granholm *et al*. reported on genetic features of their subjects, and since those with the APOE ε4 allele have a hypersensitive response to tropicamide, not accounting for this could also explain differences in results.

The pupillary response to different light conditions is also an important area of study. The pupillary darkness reflex, and the recovery time for the pupillary light response, are controlled primarily by sympathetic activation, whereas the amplitude and latency of the pupillary light response is controlled by parasympathetic activity [83]. As such, assessing the pupillary light and darkness responses, which can be done by changing light conditions, is a useful method for stimulating the pupillary changes [79], [83], [84].

Different light sources can be used to measure and assess the PLR, and the response can be influenced by the duration, spectral composition, and intensity of the light used as a stimulus [85]. The differences in stimuli determine which photoreceptor classes are activated - the rod responses, cone responses, or melanopsin-driven ipRGCs [74]. Infrared pupillometry, for example, is a method that is particularly useful when assessing the PLR, which involves stimulating the pupil with an infrared light source and then observing the response on an infrared sensor [84]. Automated infrared pupillometry can also provide a measurement of the PLR that is more reliable than using a manual flashlight to examine the PLR [86]. Additionally, chromatic pupillometry, which involves protocols using light stimuli at different wavelengths to isolate the contributions of single photoreceptors, is a

method used to characterize melanopsin retinal ganglion cells which are photoreceptive and are most sensitive to blue light at 480 nm [87].

2.5. Comorbidity of factors affecting pupillary response and risk to Alzheimer's disease

The following subsection will review the prevalent risk factors for AD and their relation, if any, to an impaired pupillary response. The comorbidity of these risk factors will also be assessed to evaluate the individual impact on the pupillary response from the individual and combined risk factors, and confounding factors will be identified and reviewed.

2.5.1. Overview of impaired pupillary responses in Alzheimer's disease

Before assessing the pupillary responses in cases involving specific risk factors to AD, it is important to assess the pupillary responses in AD patients. Much of the research into pupillary responses in AD does not assess specific risk factors and considers the impact of dementia on the PLR in general, and so this section aims to review some of this broader research before assessing individual risk factors.

There is precedence for the study of impaired pupillary responses in various physical and mental conditions including AD, to study disturbances in the parasympathetic responses relating to the pupil [53]. In patients with AD, changes relating to vision are some of the first symptoms that impact patients [88]. Potential ocular biomarkers for AD include visual acuity, contrast sensitivity, pupil reaction, colour vision, visual field, motion perception, ocular motor function, and stereopsis [88], [89]. When

assessing the pupil reaction in AD, Chang *et al*. suggest that changes are expected in the pupillary light and dark reflexes [88], the former of which will be reviewed in the following section.

A summary of AD risk factors and their impact on the PLR is shown in Table 2.2, which indicates whether an impact was identified or not, and which section each risk factor is discussed in.

Table 2.2: *Summary of AD Risk Factors and Their Impact on the PLR*

Risk Factor	**Impact on the PLR** (note: + indicates authors identified an impact on the PLR; - indicates authors did not identify an impact on the PLR; ? indicates authors identified a potential, but unconfirmed, impact on the PLR.	**Section Discussed in**
Genetics	?	4.2.1
Level of Education	-	4.2.2
Hearing Loss	?	4.2.3
Traumatic Brain Injury	+	4.2.4
Hypertension	+ (intracranial and ocular hypertension) ? (general hypertension)	4.2.5
Alcohol consumption	+	4.2.6
Obesity	-	4.2.7
Smoking	?	4.2.8
Depression	+	4.2.9
Social isolation	-	4.2.9
Diabetes	+	4.2.10
Physical inactivity	-	4.2.11

2.5.1.1. Pupillary light response in Alzheimer's disease

There are a multitude of studies that investigate the pupillary light response in AD, each of which use specific combinations of stimuli and measurement systems. Figure 2.2 shows some of the commonly used features of the PLR that are assessed in AD studies. One area of study involves assessing the resting pupil size of AD patients and comparing this to cognitively normal controls. When comparing the two groups, Kawasaki *et al*. found that the baseline pupil size in room light was significantly smaller in the AD group [90]. Frost *et al*. found similar results, finding a

smaller resting (after a 2-minute dark adaptation) and minimum pupil size after stimulus in the AD group [91]. Finally, Prettyman *et al*. found a smaller resting pupil size in the AD group compared to the control group, which they hypothesize could be caused by a sympathetic deficit caused by the loss of neurons in the locus coeruleus in AD [83].

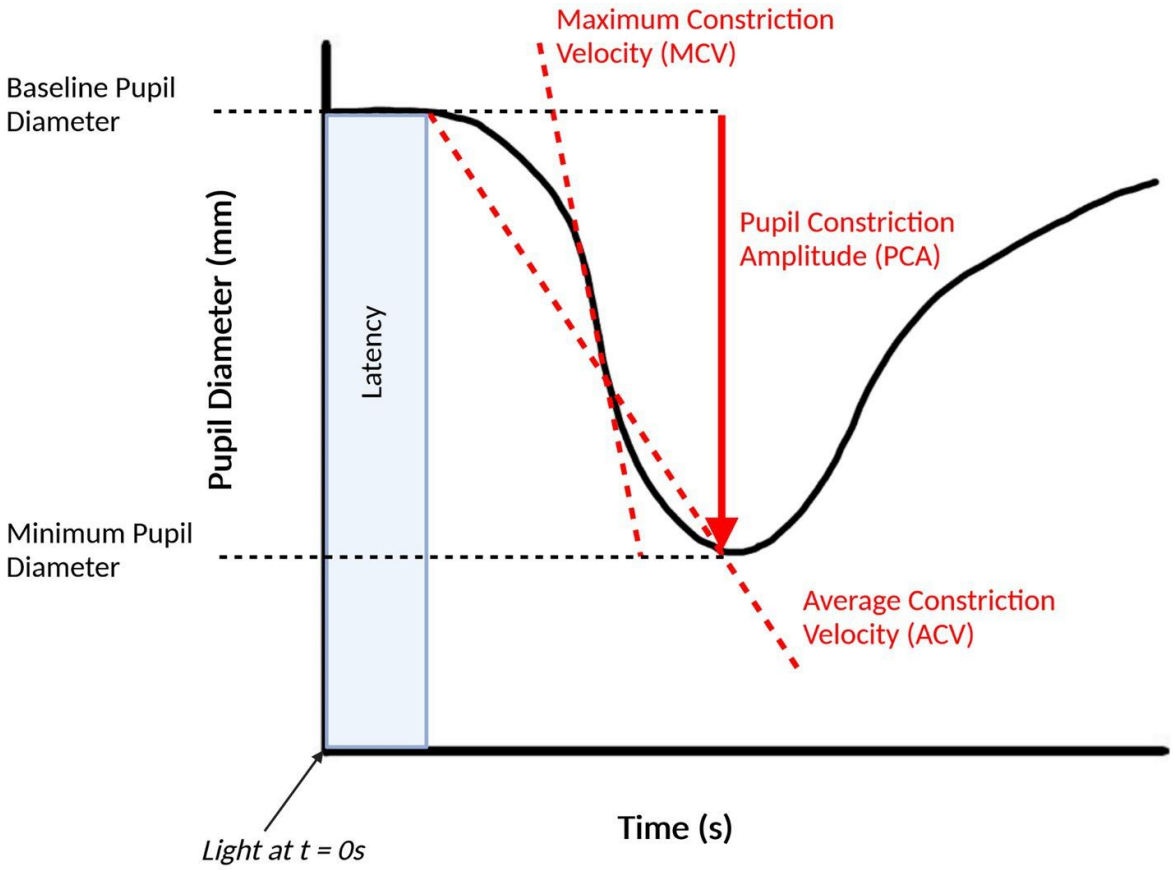

Figure 2.2: *Pupillary Light Response Plot, with commonly extracted features labelled. This figure was created by SS using BioRender.com.*

However, not all studies have replicated the studies led by Kawasaki and Prettyman. Fotiou *et al*. found no significant difference in baseline pupil size between the AD group and cognitively normal controls [92]. Additionally, Ferrario *et al*. found that the baseline pupil size was notably higher in the AD group than in the control group [93]. These discrepancies could potentially be due to different root causes of AD, which may determine whether the PLR parameters such as baseline pupil size are

impacted. As such, more research is required into an analysis of baseline pupil size in AD to obtain an accurate and reliable conclusion.

Another area of research is an analysis of the velocity and acceleration of pupil constriction or dilation in AD patients compared against cognitively normal groups. Generally, most studies of pupillometry have found a reduced maximum constriction velocity (MCV) and maximum constriction acceleration (MCA) in AD cohort when compared to healthy controls, consistent with a hypothesized parasympathetic deficiency, and have been proposed as the most accurate pupillometric parameter for differentiating AD groups from healthy controls [94]. Fotiou *et al*. observed significantly lower values for both MCV and MCA in the AD group when compared to the controls and noted that MCA was the best parameter to separate AD patients from healthy controls, with MCV in a close second [92]. Frost *et al*. similarly found reduced values for MCV and MCA in AD groups but found that MCV was the best method for classifying AD groups from controls [52]. Other studies by Fotiou *et al*. and Frost *et al*. have reported reduced MCV and MCA in AD groups [91], [95].

Prettyman *et al*. found that patients in the AD group had a reduced recovery time when compared to the control group, however they caution that this response, in conjunction with a reduced amplitude of the response, could be due to a saturation effect at the response floor, which could make it difficult to draw conclusions about how much the parasympathetic innervation of the iris plays a role in AD [83].

In contrast to most studies, Ferrario *et al*. found that the average values for MCA were higher in the AD group than in the control group and did not find a statistical difference between the MCV of the AD and control groups [93]. Although most

studies agree that MCV and MCA are reliable pupillometric markers to differentiate AD patients from healthy controls, this is not always the case – this could be due to different risk factors for AD resulting in different disease endotypes.

Other pupillometric parameters that are used in pupillometry studies involving AD are the pupil constriction and pupil dilation amplitudes. Most studies have found that AD groups have a decreased pupil constriction amplitude (PCA) when compared to healthy controls, including those led by Frost and Fotiou [52], [91], [92], [94]. Prettyman *et al*. found a reduced PCA in the AD group, but caution that just as with the reduced recovery time, that this could be due to a "floor effect" which makes it difficult to conclude that a parasympathetic innervation of the iris in AD causes these effects [83]. Granholm *et al*. found that the peak PCA was significantly reduced in AD groups when compared to healthy controls but was also reduced in Parkinson's disease patients and that there was not a significant difference between the AD and Parkinson's disease groups, which they suggest means that this test is sensitive to AD but does not have adequate specificity [82].

As with the other pupillometry parameters, not all studies have found a decreased PCA in AD patients. Ferrario *et al*. did not find a significant difference between the PCA of AD and control groups [93]. Similarly, Kawasaki *et al*. did not find any significant difference in PCA when subject to various intensities of coloured light in the AD group [90]. Van Stavern *et al*. did not find any significant difference between a preclinical AD group and the PCA [96]. As such, more research should be done to assess the validity of the PCA as a pupillometric indicator for AD.

There have been some significant relationships found between AD patients and their pupillary light responses, including their resting pupil diameters, their MCV and MCA, and their PCA. However, not all these relationships show the same reproducibility as shown by some contradictory studies. Additionally, some of these relationships are also seen in other neurodegenerative diseases, which reduces the specificity of only using these PLR metrics to separate AD patients from others. Many of these differences may be due to notable differences in the inclusion and exclusion criteria of subjects across studies, including ethnicity, sex, genetics, comorbidities and other demographics and disease endotypes. More information, including any underlying health conditions or other risk factors, should be collected from patients to be able to separate the pupillary response impacts of AD alone from the impacts from other factors, and to account for any comorbidities that may impact the response.

2.5.2. Risk factors and pupillary light response impairments

The pupillary light response appears to be impacted in AD patients; however, this measure alone lacks the required specificity to separate AD patients from healthy controls or those with other diseases. This section will review the prominent risk factors for AD and their impacts, if any, on the PLR. The aim will be to separate the individual impacts on the PLR in AD patients who may have these underlying conditions or risk factors, and to assess the comorbidity of these conditions in relation to the PLR.

2.5.2.1. Genetics

Before investigating the impact of various lifestyle and preventable risk factors for AD on the PLR, genetic risk factors will be explored – specifically, the APOE ε4 allele, and genetic factors involved in autosomal dominant AD (ADAD). There have been several studies investigating changes in the pupillary responses in subjects with genetic susceptibilities to AD – specifically when assessing the pupillary responses to light and to tropicamide.

ADAD is a rare form of AD that affects carriers with specific gene mutations, which can occur in people as young as 30 years old. The gene mutations involved in this genetic disorder primarily involve amyloid precursor protein (AβPP), presenilin 1 and presenilin 2, and mutation carriers progress to AD with 100% certainty [97]. Frost *et al*. investigated the pupil flash responses in mutation carriers (specifically in the APP 693 mutation at position 22 of the amyloid-beta fragment, APPGlu693Gln) and compared them to non-carriers, all within a single family. They found that the 75% recovery time was larger in the mutation carrier group and the percentage recovery 3.5 seconds post-stimulus was smaller in the mutation carrier group – both parameters were found to provide perfect classification of mutation carriers against non-carriers in the cohort, showing that the pupil flash response can be used in AD cases outside of the sporadic AD classification [97].

The pupillary response to tropicamide has been investigated in several studies relating to AD. A study by Higuchi *et al*. found that cognitively normal subjects with the APOE ε4 allele had a greater increase in pupil size after tropicamide-induced changes, which they suggest shows that this hypersensitivity can be seen in APOE ε4

carriers before the onset of AD [81]. These findings agree with a study by Turana *et al.*, which found that subjects with the APOE ε4 allele had the highest pupillary hypersensitivity response when a drop of 0.01% tropicamide was put on their eye, when compared to the other subjects, and suggested that a combination of biological and clinical markers is required to increase the positive predictive value towards amnestic mild cognitive impairment cases [98].

It is evident that there are some genetic factors that are associated with different pupillary responses. However, more research should be done on the PLR in APOE ε4 carriers and carriers of specific genetic mutations to make any conclusions about how these factors influence the PLR.

2.5.2.2. Level of education

A lower cognitive reserve leads to vulnerability to cognitive decline [99], as cognitive reserve assists in maintaining brain function [100]. Individuals with a higher cognitive reserve have a later onset of cognitive functions being impacted by AD or age-related pathology, as their higher cognitive reserve can tolerate more pathology [101]. Thus, increasing a person's cognitive reserve can assist in preventing dementia. The level of education that a person receives, and their occupational status, are both factors that contribute to increasing the brain's cognitive reserve [99]. Cognitive ability increases with education before plateauing in late adolescence with few further improvements with education after an age of 20 years [102] – thus, cognitive stimulation is especially important in early life to assist in building cognitive reserve. Stern *et al*. also found that participants in low occupational levels throughout their lifetimes, based on the United States census categories, have a greater risk of

developing dementia [101]. Other cognitive activities in adulthood, including reading, playing games, playing music and creating art, speaking multiple languages, and participating in leisure activities also assist in maintaining cognition [99], [101].

To the authors' knowledge, no study has published any results that directly link the subject's level of education or cognitive reserve with their pupillary responses, either to light or another stimulus. Research should be done to consider the direct impacts of education and cognitive reserve on the PLR both dependent and independent of AD to potentially aid in diagnosis, given that an increase in cognitive reserve can delay the onset of symptoms of AD and make a diagnosis more challenging [101].

2.5.2.3. Hearing loss

Hearing loss at any scale, including mild hearing loss, increases the long-term risk of cognitive decline and dementia [103], [104], [105], [106], [107], [108], [109], [110], [111]. In many cases, hearing loss can predate and predict a clinical diagnosis of dementia [112], and auditory scene processing deficits could be considered a functional marker for AD pathology [113]. According to Livingston *et al.*, hearing loss had the highest population attributable fraction of potentially modifiable risk factors for dementia, and with every 10 dB of reduction in hearing a decrease in cognition is found, potentially due to reduced cognitive stimulation [6]. However, these findings are only consistent with people who do not use hearing aids – hearing aid use is one of the largest factors that can protect against the onset of dementia [6].

Previous functional MRI (fMRI) studies conducted at resting state have shown a reduction in spontaneous neural activity in hearing loss patients which correlated with a reduction in cognitive performance [114]. There are several possible

mechanisms to explain the relationship between hearing loss and dementia, which are defined by Griffiths *et al*. as: common pathology affecting the cochlea and ascending pathway (causing hearing loss) and the cortex (causing dementia); impoverished environment causing decreased cognitive reserve; a requirement for increased cognitive resources for listening; the interaction between brain activity related to auditory cognition and dementia pathology [115].

General pupillary responses arising from an increase in mental or cognitive effort have been investigated in hearing loss subjects [116], [117], [118]. However, in their 2016 systematic review, Wang *et al*. did not identify any results for studies directly linking the pupil light reflex and hearing impairment [68]. However, they did investigate hearing impairment and its associations with the parasympathetic response, which has been shown to be linked to the PLR [68]. Hasson *et al*. found a negative correlation between hearing problems and parasympathetic activity and associated an increase in hearing problems with a decreased ability to "unwind" or recover from the stress due to diminished parasympathetic activity [119]. Mackersie *et al*. found that subjects with hearing loss had greater stress-related autonomic nervous system activation and noted that an important aspect of a stress response could include activation of the sympathetic branch and suppression of the parasympathetic branch [120].

No direct relation between the PLR and hearing ability or loss was found in the search conducted, and minimal evidence was shown to link a decreased parasympathetic response to hearing loss. Despite this, as the PLR is governed by sympathetic and parasympathetic activity, this potential link between hearing loss and a decrease in

parasympathetic activity should be explored in further research, using the PLR as a metric.

2.5.2.4. Traumatic brain injury

Traumatic brain injury (TBI), including mild and severe injuries, is a known risk factor for AD [121], [122]. In particular, a single, severe, TBI is associated with widespread hyperphosphorylated tau pathology in both humans and mouse models [123]. The risk of developing AD due to TBI increases with both the severity of the injury and the number of injuries sustained, and the risk of dementia is stronger closer to the time that the injury occurs which can lead to early-onset AD in some people [122]. Notably, those with a higher occupational risk for head and brain injuries are more likely to develop AD as a result of their increased likelihood of these injuries [124], [125], [126]. Many athletes including boxers, American football players, ice hockey players, soccer players, rugby players, and wrestlers, in addition to military veterans, have had associations with chronic traumatic encephalopathy (CTE), a progressive neurodegenerative disease which is associated with repetitive TBI experienced in sports and military activity and is a risk factor for dementia [127].

There have been several studies investigating the PLR in TBI subjects, through several different methods of classifying TBI. A common consequence of TBI is an increase in intracranial pressure (ICP), which is one such parameter used to characterize TBI [128]. Chen *et al*. used an algorithm to characterize the pupillary response relating to ICP, called the Neurological Pupil index (NPi), which takes in common parameters of the PLR including the pupil's minimum and maximum sizes, constriction percentage and velocity, and dilation velocity [129]. They found that subjects who had decreased

PLRs had higher peaks of intracranial pressure, using the NPi to characterize the pupillary responses [129]. Another means of assessing TBI is through the Glasgow Coma Scale (GCS), which can be used to measure the neurologic status of patients, with more severe brain injuries being classified as 8 or less on this scale, and mild to moderate brain injuries being classified as 9 or more [130]. Park *et al*. found that diminished PLRs were associated with a lower GCS score and found that the initial NPi value of the group of subjects receiving a "poor" prognosis was lower than the group with a "favourable" prognosis, demonstrating the potential for the PLR to be used in diagnosing and classifying TBI severity [130].

Several other studies have shown direct links between TBI and the PLR, showing that multiple parameters of the PLR are reduced in magnitude after TBI, particularly when using monocular test measurements [131], [132]. Most studies assess the PLR in the short term after TBI occurrence and do not follow-up on long term PLR changes, although Truong *et al*. found PLR impairments in mild TBI patients in the chronic recovery phase (greater than 45 days post-injury) when compared to normal controls [131]. As such, there is sufficient evidence to show that TBI is associated with noticeable changes in the PLR, at least in the short term. More research should be done to investigate how these changes are affected in the long term.

2.5.2.5. Hypertension

Hypertension, specifically persistent hypertension in midlife, is associated with an increased risk for dementia in later life. An elevated systolic blood pressure in midlife has been shown to increase dementia risk, with the risk increasing if this hypertension continues later in life [133]. A potential mechanism for how this

contributes to dementia risk is through alterations of regulatory mechanisms of the cerebral circulation, which compromise the blood supply to the brain [134]. Additionally, midlife hypertension is associated with reduced brain volumes and an increased white matter hyperintensity volume [135]. However, this risk can be reduced when anti-hypertensive medications are taken [136].

Hypertension has been linked to an altered PLR in several studies, however most studies focus on either intracranial hypertension or ocular hypertension. Grozdanic *et al*. have found, in separate studies, that the PLR is reduced in rats after acute elevation of intraocular pressure [137], [138]. In human subjects, reduced amplitudes of the PLR have been associated with increased intracranial pressure and in intracranial hypertension including idiopathic intracranial hypertension and is often used to monitor neurocritical care patients [86], [129], [139], [140], [141].

Importantly, hypertension can affect the autonomic nervous system, which is characterized by sympathetic and parasympathetic activity. Multiple studies have shown that patients with borderline hypertension display an increase of sympathetic activity [142], [143], [144], [145], and a decrease of parasympathetic activity [142], [145]. Although not many studies have directly evaluated the effects of general hypertension on the PLR, its link to the sympathetic and parasympathetic activity shows that further research should be conducted in this area to investigate a link between the PLR and general hypertension.

2.5.2.6. Alcohol consumption

Heavy drinking has been associated with cognitive impairment and dementia [146], however due to its complex entanglement with sociocultural and health-related

factors, it is challenging to fully understand how alcohol alone contributes to dementia risk [6]. Venkataraman *et al*. suggest that alcohol misuse, such as binge drinking or chronic alcohol use, could lead to neuroinflammation and neuronal cell death, which could be a mechanism for how alcohol consumption increases AD risk [147]. Additionally, higher alcohol consumption has been associated with an increased risk of hippocampal atrophy, which is considered a specific marker of AD [148]. Regardless of the mechanisms involved, moderating or reducing alcohol intake can reduce the risk of AD [146].

The parasympathetic response may be impaired in alcoholics, due to lesions in the parasympathetic supply [149]. This has been shown to manifest as an impaired PLR when comparing alcoholics to non-alcoholics [150], [151]. Rubin also compared alcoholics who abstained from drinking one month prior to the study to alcoholics who did not abstain and found that both groups had an equally defective rate of pupillary contraction, but the alcoholic drinkers had a slower rate and amplitude of dilation, showing that alcoholics demonstrate an impaired parasympathetic outflow regardless of their drinking activity, but that the sympathetic deficiency is dependent on whether the alcoholic abstains from drinking for an extended period of time [150].

Changes in the PLR are not only observed due to consistent alcohol consumption over an extended period but are observed during the act of consuming alcohol or while a person is actively drunk. Short term alcohol consumption leads to dilated pupils and slower pupillary reactions [152]. Studies have shown that the PLR may be a good measure to classify a person's current inability to work or drive due to alcohol consumption or sleep deprivation [153], [154]. As such, it is important to separate

the instantaneous impacts of alcohol consumption from the more prolonged impacts from alcoholism or heavy drinking on the PLR, and more research should be done to separate the two.

2.5.2.7. Obesity

An increased body-mass index (BMI), specifically in the obesity-defined range, is associated with an increased risk of dementia [155]. Additionally, increased adiposity is related to AD, potentially due to increased vascular stress, however the exact mechanisms for this are still unknown [156]. Further, the risk of dementia has been shown to vary with the age of onset of obesity, with a higher risk associated with younger adults with obesity when compared to adults who only develop the condition later in life [157]. While there is data that supports the claim that weight loss in obese and overweight adults is associated with improvements in performance across multiple cognitive domains [158], according to Livingston *et al.*, there is no data specific to the long-term effects of weight loss in overweight and obese adults in lowering dementia risk [6].

There is limited research that assesses the links between obesity and the PLR, and the limited research presents conflicting results. Baum *et al*. found a decreased PLR with an increased BMI in children and adolescents [159]. Blüher *et al*. assessed changes in the PLR of obese children, after exercise and lifestyle interventions were made to decrease BMI and found that reductions in BMI were associated with a higher dilation velocity, higher relative light reflex amplitude, and higher constriction velocity [160]. Within a group of healthy adults with a range of BMIs ranging from normal to obese, Segal *et al*. found that those with a higher BMI also had a higher

average dilation velocity post-stimulus and concluded that BMI levels positively correlate with sympathetic activity [161]. When investigating sets of identical twins who had different BMIs (obese and non-obese classifications), Piha *et al*. did not find significant differences in heart rate, blood pressure, or pupillary responses between the obese and non-obese twins and concluded that neither sympathetic nor parasympathetic responsiveness is significantly affected by obesity and instead is affected significantly by genetic factors [162]. These differences may be due to the different subject selection for these studies, including variability in age and genetics. Additionally, these changes could be due to other risk factors or comorbidities that may accompany obesity, such as physical activity levels and other lifestyle aspects.

No other significant study comparing obesity, BMI, or other weight-related factors to the PLR was found in the search. The studies that have been published to date used different age and genetic groups and have all presented different results, and so no conclusion can be made. Further research should be done with broad ranges of age and genetic groups to investigate any potential relationship between obesity and the PLR.

2.5.2.8. Smoking

Smokers are at a higher risk of developing dementia when compared to non-smokers [163], [164], [165], [166]. However, smokers have a higher risk of premature death which could occur before their age of dementia onset, so these competing risks may introduce biases and discrepancies in the association between smoking and dementia risk [167], [168]. Regardless, not smoking can increase life expectancy and health, and stopping smoking can reduce the dementia risk [169]. Exposure to smoke

through second-hand smoke is also associated with more memory deterioration [170], although limited literature exists in this specific area [6].

To the authors' knowledge, no study has published any results that directly link long-term nicotine smoking to an impaired PLR. However, there have been studies that have investigated the relationship between nicotine and smooth muscle function. Studies have shown that nicotine may act on vascular smooth muscle and induce vascular relaxation in rats [171] or vascular contraction or relaxation in humans [172]. Further, nicotine may alter vascular smooth muscle cell phenotypes [173], [174]. Because the function of vascular smooth muscle cells may be a biomarker for AD, and these vascular smooth muscle cells may undergo phenotypic transitions in AD [175], this supports the need for further research in this area.

Additionally, there have been studies linking smoking and intraocular pressure. Mansouri *et al*. found that chronic long-term smokers had a higher mean intraocular pressure than non-smokers [176]. Similarly, Lee *et al*. found that current smokers had a slightly higher mean intraocular pressure than the non-smokers in their study [177]. Although not directly linked to the PLR, this change in intraocular pressure should be investigated further to determine whether it, in turn, causes a change in the PLR in smokers.

2.5.2.9. Depression

Having depression is associated with an increased risk of dementia and AD [178], [179]. Depression is also part of the dementia prodrome and can be seen in the early stages of the disease, and thus there has been debate as to whether depression is only a symptom of dementia, or if it is an independent risk factor for dementia [179].

The mechanisms involving depression and AD are likely to be multifactorial and may include vascular and neuropathological mechanisms [180]. Some molecular mechanisms, such as chronic inflammation, are common in the pathogenesis of both major depression and AD [178]. Livingston *et al*. have not found conclusive evidence for the difference between treated and untreated depression regarding the risk of dementia [6]; however, the use of antidepressants has been shown to improve amyloid beta clearance [181].

Some studies have investigated the PLR in subjects with depression, in various capacities. The PLR has been shown to be altered in patients with major depressive disorder (MDD) when compared to controls [182]. Mestanikova *et al*. found that the PLR was diminished in the left eye of adolescent girls with depression, but not in the right eye [183]. Berman *et al*. found that the PLRs were diminished in depressed patients both with and without a seasonal pattern, when compared to healthy controls [184]. Further, Fountoulakis *et al*. found that subjects with depression had a shorter latency for pupil constriction post-illumination, when compared to healthy controls, which suggests a norepinephrine hypoactivity in melancholic depression [185].

Some researchers have assessed how the PLR can be impacted by the conditions that the depressed subjects are under. Bar *et al*. found that the PLR was impacted by antidepressant use – they found that acutely depressed patients who had not taken antidepressants did not differ significantly in PLR parameters, other than relative amplitude, compared to healthy controls, although those taking antidepressants had significant changes in their parasympathetic function [186]. Feigl *et al*. investigated

how the mean daylight exposure could impact the PLR in subjects with mild and moderate non-seasonal MDD but found no significant differences between the MDD subjects and healthy controls regardless of the daily and hourly light exposure including recommended light therapy that is recommended for MDD patients [187]. However, Laurenzo *et al*. found that in addition to the PLR being impacted in MDD subjects when compared to healthy controls, MDD subjects displayed reductions in the post-illumination pupil response to high-intensity blue light, which was less pronounced in months with fewer daylight hours [188]. This may be due to the difference in methods and stimuli used or due to other differences in subject inclusion criteria. More research should be done to investigate impaired PLRs in depressed subjects of various ages and under various conditions.

Although considered to be a separate risk factor from depression, aspects of social isolation have several similarities with depression. However, to the authors' knowledge, no study has published any results that link the subject's level of social engagement with their pupillary responses, either to light or another stimulus, and thus the social isolation risk factor has not been further explored in this review.

2.5.2.10. Diabetes

Having diabetes is a significant risk factor for dementia and AD [189]. It is thought that diabetes, specifically type 2, could increase the risk through insulin resistance, impairing glucose metabolism in the brain [190]. Dementia risk is higher with increased duration and severity of diabetes, but the effects of diabetic medications on dementia outcomes or cognition are unclear [6]. It is generally agreed that type 2

diabetes is a risk factor for the future development of dementia, however specific treatment for diabetic control has not been shown to decrease dementia risk [191].

There are extensive studies that have assessed the relation between diabetes and the eye, including several aspects of the PLR. Lanting *et al*. found that overall, the diabetic patient groups studied had a higher PLR latency when compared to healthy controls and normal values and claims that this represents parasympathetic dysfunction [72], [192]. A study by Bista Karki *et al*. also supported the concept of parasympathetic dysfunction in diabetic patients, with results that showed that the diabetic subjects had a lower maximum and mean constriction velocity, lower constriction amplitude, and a lower relative reflex amplitude when compared to the healthy controls [33]. Ishibashi *et al*. subjected diabetics and healthy controls to both red and blue light, and found that with both light colours, the pupil constriction was slower and less pronounced in the diabetic group when compared to the healthy control group [193]. Karavanaki *et al*. compared diabetic children with healthy children and found that the diabetic group had impaired pupillary adaptation in the darkness [194]. Several studies that found a reduced PLR in diabetic patients when compared to healthy subjects, with further reductions when comparing diabetics with autonomic neuropathy and without, suggested that pupillometry could help to identify diabetic autonomic neuropathy [195], [196], [197].

Not all studies support the claim that the PLR is significantly different between diabetic and non-diabetic subjects. Lerner *et al*. noted that although there were some differences in pupillometry values, that most had poor accuracy as a screening tool due to inadequate specificity and sensitivity [45]. Hreidarsson and Gundersen

found that in type 1 diabetics who had a normal or near-normal sensory pathway, there was no significant difference in latency or other PLR parameters when compared to healthy controls with the same pupil size, and only a minor reduction in response amplitude [198].

Overall, there are multiple studies that show a reduction in the PLR among diabetic subjects when compared to healthy controls. Pupillometry has been suggested as a diagnostic tool for the monitoring of diabetes progression, specifically when assessing the development of certain side effects including autonomic neuropathy. However, there is a potential confound with diabetic retinopathy, where pupillary abnormalities may precede a diabetic retinopathy diagnosis [33], and thus it is difficult to know whether any impairments to the PLR are due to the retina or due to nervous system defects. Additionally, there could be confounding effects with comorbidities that may exist with diabetes, which could also be different in type 1 and type 2 diabetes cases.

2.5.2.11. Physical inactivity

Physical inactivity is a risk factor for dementia, and older adults who exercise regularly have a better chance of maintaining cognition [199]. Being physically active is considered a protective factor against cognitive decline [200], [201]. Livingston *et al*. highlight that although physical inactivity is considered a separate risk factor for dementia, there are several overlaps between physical activity and other risk factors such as obesity and diabetes, and confounding factors exist with age, sex, social class, and cultures [6]. To the authors' knowledge, no study has published any results that link the subject's level of physical activity with their pupillary responses,

either to light or another stimulus, and thus this risk factor will not be explored further in this review.

2.5.3. Confounding factors

As was highlighted earlier, pupillary responses can be evoked due to a variety of factors and stimuli. Outside of the non-genetic and lifestyle-related risk factors for AD outlined by Livingston *et al*., there are other factors that can impact AD risk and the PLR. Additionally, some of the AD risk factors have a comorbidity with one another, which could confound potential relationships between the individual risk factors, AD, and the PLR. Some of the prevalent confounding factors will be outlined in this section.

2.5.3.1. Changes to the eye and AD

When assessing the PLR, it is evident that changes to the eye itself may impact the response, including retinal changes. There are many studies that have related vision changes to AD, as the eye is closely related to the brain – the retina shares important pathways, both structural and pathogenic, with the central nervous system [202]. AD may impact visual function early in the disease progression, and losses in visual function correlate with cognitive losses [203]. Rogers and Langa found that generally, poor vision that is left untreated is associated with cognitive decline and AD [204].

There are also associations when considering, more specifically, changes to the retina and AD. Amyloid-beta and phosphorylated tau can accumulate in the retinas in early-stage cases of AD, which could be used as an early biomarker for AD [26], [205]. The retinal nerve fibre layer thickness has been found to be smaller among AD patients when compared to healthy controls [26], [206], [207], as has the retinal

ganglion cell layer, inner nuclear layer, and outer nuclear layer [207]. Cabrera DeBuc *et al*. suggest that retinal geometric vascular and functional parameters could be associated with retinal changes due to cognitive decline and could serve as a useful clinical marker of cognitive decline [202].

It may be difficult to separate these changes in the eye associated with AD, from independent changes to the parasympathetic or sympathetic response resulting in an impaired PLR. It could be claimed that retinal degeneration and other retinal and optic nerve changes in AD could be, at least partially, responsible for the reduced PLR observed in AD; however, this is not supported by clinical observations of AD patients in a neuro-ophthalmological examination [88]. Further research should be done to separate ocular and retinal changes to the PLR from direct AD-related changes.

2.5.3.2. Population demographics and PLR

There are several demographics of a population that can influence the PLR. The specific factors that will be highlighted are age, sex, and living environments.

Age

There are pupillary changes that occur with age in otherwise healthy adults. Many studies have shown that after growing until adolescence, the size of the pupil decreases with age [50], [67], [208], [209], [210], [211]. In terms of the PLR, Sharma *et al*. found that the amplitude of the PLR to blue light was reduced with age [208]. Bitsios *et al*., using green light, and Fotiou *et al*., using white light, similarly found a reduction, among older subjects, in the PLR using green light, but did not find a difference in the latency [209], [210]. This contradicts Feinberg and Podolak's

conclusion that pupillary latency increases with age [211]. Although there are disputes as to whether pupillary latency changes with age, the finding that pupil size decreases with age is an important factor to consider when assessing the amplitudes of the PLR.

Sex and Gender

Sex and gender differences have been identified in AD prevalence, clinical manifestation, and prognosis [212]. Women have a higher lifetime risk of developing AD compared to men [213], [214], however men have a shorter lifespan after diagnosis [215].

Fan and Yao found that females have a higher parasympathetic activity and lower sympathetic activity when compared to males, consistent with findings presented in other cardiovascular studies [216]. Van Stavern *et al*. found that there is a potential effect from an individual's sex that could influence the PLR – an example provided is that males with a biomarker showed a reduced constriction percentage when compared to males without biomarkers, although it was not found to be statistically significant [96]. Further studies should be conducted to assess sex and gender difference in AD risk factors and the PLR, to draw adequate conclusions.

Living Environment

An individual's living environment can also affect AD risk, in addition to the PLR. Livingston *et al*. identified air pollution as a risk factor for AD, likely due to vascular mechanisms [6]. Carey *et al*. investigated both air and noise pollution and found that higher levels of air and noise pollution correspond with higher risks of dementia [217]. Interestingly, Paciência *et al*. found that the walkability of school

neighbourhoods was negatively associated with the pupillary response, specifically with the pupil constriction amplitude and redilation time, which they suggest is due to the school environments affecting the lung function of students, an effect which may be partially mediated by the autonomic nervous system [218]. As shown, there are several aspects of an individual's living environment that can increase risk for AD and can impact the health of residents, however more research should be done to assess other living environment parameters with AD risk and the pupillary light response, while controlling for other factors.

2.5.3.3. Comorbidity between Alzheimer's disease risk factors

This review aimed to distinguish individual risk factors and their specific contributions, if any, to an impaired PLR. However, many of these risk factors have comorbidities, and as such it can be difficult to distinguish individual impacts. Some of the prevalent comorbidities will be discussed here.

There are several diseases and conditions that share risk factors with dementia, and conditions that may be side effects of dementia. Those living with dementia may not remember to tell family members or health professionals their symptoms, or may struggle to follow health and nutrition plans, which could increase infections [6]. A reverse causation between dementia and depression can also exist, where depressive symptoms may result from dementia neuropathology [6]. Prodromal dementia may also stop people from exercising, and so physical inactivity, like depression, may either be a consequence or cause of dementia [6]. Additionally, many of the risk factors of dementia are also risk factors for cardiovascular diseases including hypertension, obesity, and diabetes [219], all of which are individual risk

factors for the disease itself and have been shown, to varying extents, to have some impact on the PLR.

In terms of the relationship between risk factors, there are many. Several studies suggest that hearing impairment is associated with psychosocial problems, including depression or loneliness [68], [220], [221], [222]. Considering that depression and social isolation are additional risk factors for AD, this indicates a significant comorbidity. The ability to communicate with people depends significantly on hearing ability, and so hearing impairment can have a significant impact on social life, leading to social isolation and then, subsequently, to depression, cognitive decline, and dementia [112], [223]. Further, the prevalence of hearing impairment is more common in diabetics than in non-diabetics, and Bainbridge *et al*. suggest that hearing impairment may be an under-recognized complication of diabetes [224].

Diabetes and obesity are closely linked. Both conditions have a pathophysiology that is attributed to insulin resistance and insulin deficiency [225]. Obesity is associated with an increased risk of diabetes and is also associated with other health conditions including high blood pressure, high cholesterol levels, arthritis, and asthma [226]. Physical activity is shown to be an important method of combating both diabetes and obesity, which further relates these risk factors [225]. Additionally, Mokdad *et al*. found that adults with less than a high school education had the highest rate of diabetes among all educational levels [226].

Education, understandably, can inform an individual's social and lifestyle habits. Helliwell and Putnam state that education is usually the most important predictor of

social engagement [227]. It is thus important to consider how education may be related to risk factors related to social engagement and, by extension, depression.

2.6. Concluding remarks

AD is rapidly increasing around the world, and the current aim is to prevent the onset of the disease. The pupillary light response has been shown to be impaired in current AD subjects, but it is currently unknown if it could be used as a tool in at-risk groups to predict AD risk. This review outlined prevalent AD risk factors and assessed the pupillary light responses evoked in AD subjects and those belonging to AD risk factor groups.

Traumatic brain injury, ocular and intracranial hypertension, alcohol consumption, depression, and diabetes are all AD risk factors that have demonstrated changes in the PLR, in varying time frames. Hearing loss, smoking, and genetic factors have had associations with changes to the parasympathetic activity, which could indicate an impaired PLR, however further research should be done to confirm this hypothesis. Genetic risk factors have additionally had limited direct associations with the PLR, and more research should be done to investigate this relationship. No conclusions could be drawn between level of education, social isolation, obesity, and physical inactivity and the PLR, mainly due to a lack of literature, so more research should be done to investigate any potential relationships. Further, there is a comorbidity between some AD risk factors, therefore further research is necessary to separate the individual impacts of these risk factors on the PLR.

With these findings, the PLR has clearly been shown to be impaired in current AD patients and in certain at-risk groups. Currently, pupillometry using the PLR has

value as a confirmatory measure of AD. In the future, the PLR has the potential to be used as an early diagnostic tool for AD and could be used, in conjunction with a lifestyle, life-event, genetics, and physiological assessment, to identify when preventative measures should be taken for AD. To enable this, future research in this area should consider the impacts of individual lifestyle, genetic, life-event and physiological risk factors for AD and how these relate, if at all, to the PLR. Further, these future studies should ensure that a standard methodology for pupillometry measurements, as was proposed by Kelbsch et. al. [74], should be used to ensure that these studies can be easily compared.

Chapter 3: Comparison and development of pupillary light response tools and protocols

This chapter has two main aims: to compare the pupillometers that the project team has access to, and to determine the most reliable PLR protocol and equipment. This chapter presents the first data collected after receiving ethics approval from the University of Oxford (CUREC 2, Ethics Approval R58903/RE001), which will be essential when conducting later work where pupillary data will be collected and used in large quantities.

This chapter includes background information on photoreceptors and their contributions in the pupillary light response, a presentation of different pupillometers tested, a comparison of different testing conditions, and a comparison of different pupillometry protocols. Further, it outlines the testing data used for PLR methodology selection and defines issues that were discovered through testing and validation stages, with proposed mitigation methods. Importantly, the data collected and presented in this chapter informs the PLR metrics used in later chapters for comparison with CVR and neurodegeneration risk classification.

This chapter relates to Research Question 2, and includes work from the following published abstract:

Sparks, S; Hayes, G; Pinto, J; Martin, J; Spitschan, M; Bulte, DP (2024). "Comparing the pupillary light response using two pupillometers with cerebrovascular reactivity". Oxford Ophthalmological Congress 2024. Published abstract. Poster presentation.

3.1. Introduction

Chapter 2 presents an introduction to the parasympathetic and sympathetic pathways that contribute to pupillary constriction and dilation, mediated by the sphincter and dilator muscles. Before analysing different ways to elicit pupillary responses to light, beyond this introduction, a fundamental understanding of photoreceptors and other contributors to the pupillary light response is necessary.

3.1.1. Photoreceptors

Photoreceptors are cells in the retina that respond to light, which contain light-sensitive molecules, opsins [228]. Photoreceptors, in conjunction with the dilator and sphincter pupillae muscles, contribute to the PLR, in both the short-term and steady state responses [18], [229]. Each photoreceptor class has different contributions to the PLR, and there are three main classes of photoreceptors: cones, rods, and melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs).

Each type of cone (short, middle, and long wavelength cones – S, M, L cones), rods, and melanopsin all have different spectral sensitivity levels. Martin *et al.* discuss these levels, shown below in Figure 3.1 retrieved from PyPlr documentation [18].

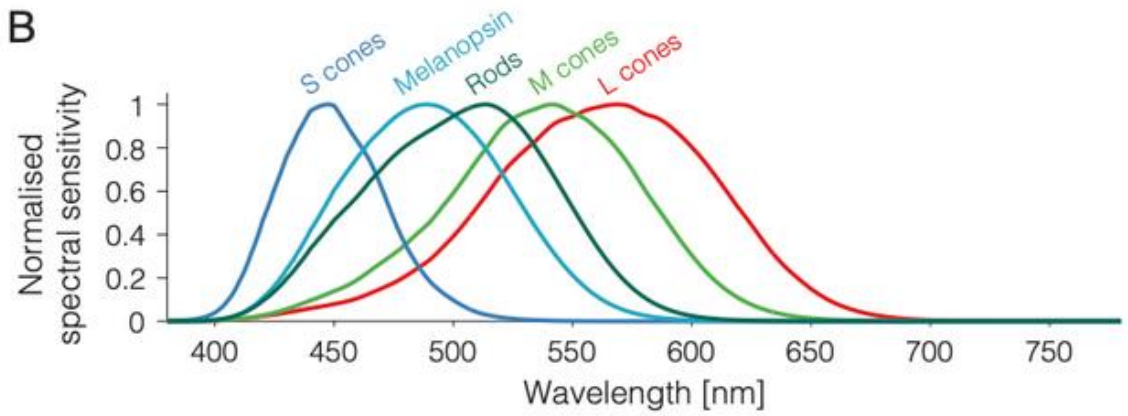

***Figure 3.1**: Spectral Sensitivity Levels of Wavelengths of Light/ Retrieved from PyPlr documentation* [230].

Rods and cones dominate in the shorter term pupillary responses to light [231], while ipRGCs contribute to sustained pupillary constriction in response to light [232], particularly near the peak melanopsin spectral sensitivity of 480 nm [49]. Rods and cones hyperpolarize membranes, while ipRGCs cause membrane depolarization [233]. Even with their similarities, rods and cones contribute different responses – for light intensities below the threshold of activation for melanopsin cells, the PLR is dominated by rods with minimal or no input from cones [234]. There are significant differences in the contributions of rods, cones, and ipRGCs to the pupillary light response that must be considered when assessing the pupillary light response. Lucas *et al.* outline how different photoreceptors contribute to the pupillary light response, demonstrating that the different retinal photoreceptors are upstream of circadian, neuroendocrine, and neurobehavioral responses to light [235].

In the methods, the aim was to assess as many different photoreceptors as possible, to aim for the impact of photoreceptors on pupillary light responses to be

disentangled from smooth muscle or other contributions from the autonomic nervous system in future work.

3.1.2. Factors Influencing Pupil Size

As discussed in Chapter 2, there are several physiological and autonomic factors that control pupil size. In addition to age, the autonomic nervous system, the smooth muscle of the iris (all discussed in Chapter 2) and the contributions of different photoreceptors, there are other factors that can influence pupil size. Some of the most prevalent factors influencing pupil size are summarized in Table 3.1.

***Table 3.1**: Factors influencing pupil size*

Factor	Mechanism
Age	Pupil diameter decreases with increasing age [208].
Autonomic Nervous System	The sympathetic nervous system contributes to pupillary dilation, while the parasympathetic nervous system contributes to pupillary constriction [15].
Smooth Muscle of the Iris	The sphincter muscle, controlled by the parasympathetic nervous system, controls pupillary constriction. The dilator muscle, controlled by the sympathetic nervous system, controls pupillary dilation [15].
Photoreceptors	Rods, cones, and melanopsin contribute to the PLR, with variable relative contributions, depending on stimulus intensity, duration, and spectral content [235].
Light Adaptation Level	Pupil area decreases with increasing irradiance, approximately over a 9 $\log_{10}$ intensity range [235]. Increasing or decreasing the time spent in adapting to the dark can influence pupil size [236].
Depth of Focus	Pupil size affects visual resolution and the depth of focus, with a smaller pupil size increasing the depth of focus [237].
Arousal	Linked to the sympathetic nervous system, pupil size increases with increased autonomic arousal [238].
Fatigue	Increased fatigue has been reported to decrease pupil size, with increased fatigue often corresponding to decreased arousal [239].
Consensual Responses	When only one eye is exposed to light, there is a consensual response in the second eye, where the second eye reacts in symmetry with the stimulated eye [240].
Pharmacological Influences	Various pharmacological agents may influence pupil size. These include pupil dilators such as tropicamide (often used before comprehensive eye exams or ocular procedures [241]) and caffeine [242], and pupil constrictors such as opioids [243].

3.1.3. Equipment Available

3.1.3.1. Neuroptics

The NeurOptics PLR-3000 (NeurOptics, Irvine, CA) is an established research tool that has been widely used in research and clinical practice. It is a handheld pupillometer that uses an infrared camera to capture and measure pupillary size and dynamics, which can assist with more precise measurements of the PLR [244].

The NeurOptics PLR-3000 has several settings that can be adapted to suit various research purposes. This includes the types of stimuli (positive stimulus, negative stimulus, extended mode, and static stimulus), the intensity of the light stimulus (0 µW, 1 µW, 10 µW, 50 µW, 121 µW, 180 µW), and the timings of stimuli (ranging from millisecond to seconds scale).

3.1.3.2. PyPlr

PyPlr is an open-source combination of hardware and software designed in-house [18]. It has ten LEDs of different wavelengths across the visible light spectrum, which allows much more flexibility in the spectrum of light that can be used. Additionally, the timings can be adjusted, along with the LED intensities ranging from 0 to 1.8×10^4 cd/m^2 according to initial scoping measurements [18]. This allows for greater flexibility in administration of light protocols, which is desirable for the future purpose of assessing various parameters of the pupillary light response and the post-illumination pupil response (PIPR) and their potential relationship to metrics of brain health, and to potentially disentangle the relative responses from different types of photoreceptors as well as the smooth muscle.

This device has previously been tested against the NeurOptics PLR-3000 using a white flash protocol [18], and has been tested using a blue and red stimulus protocol as a baseline. Further data in a larger cohort is required to validate its use in this research.

3.1.3.3. 4YPLR

The 4YPLR, an in-house pupillometer designed and built by J Rosser who was a former 4th year engineering project student of Professor Bulte, has not been tested in-lab yet; preliminary results from Rosser's report show that the 4YPLR can stimulate and measure pupil contraction and dilation post-illumination. However, this device did not provide any additional information that could not be collected from the NeurOptics, and since the NeurOptics showed promising preliminary results, it was decided to not test the 4YPLR further, to ensure that results could be compared to and validated against existing studies using the same equipment.

3.2. Methods

Using both pupillometers, various protocols were established to determine the most effective protocol for assessing the PLR and PIPR. Protocols were chosen based on the available equipment to the study, previous literature demonstrating relationships between neurodegeneration and parameters of the PLR in a multitude of protocols as was introduced in Chapter 2 [245], and the requirement to have a range of protocols to potentially isolate the contributions of different photoreceptors and branches of the autonomic nervous system.

From the literature review in Chapter 2, changes to both parasympathetic and sympathetic activity are shown in at-risk groups for dementia. As such, choosing

protocols that elicit responses from both branches of the autonomic nervous system is essential – this can be achieved by assessing the constriction and dilation phases of a positive stimulus of white light. Additionally, changes in the PIPR in response to chromatic pupillometry have been shown in AD cases [246], motivating the use of chromatic stimuli in this work. Ultimately, limited research currently exists that links the PLR and PIPR to at-risk groups for dementia, either through elevated lifestyle risk, genetic risk, or intrinsic risk potentially mediated by neurovascular risk factors. As such, it is important to consider a multitude of protocols to potentially identify trends between these at-risk groups and various parameters of the PLR and PIPR in later chapters. Protocols assessing both the PLR and PIPR have been included in this preliminary testing, each with a specific interstimulus interval (ISI).

For each experiment, all participants provided informed written consent before each session, and the study was approved by the Medical Sciences Interdivisional Research Ethics Committee (MS IDREC) of the University of Oxford's Central University Research Ethics Committee (CUREC).

Table 3.2 outlines the protocols tested in this chapter.

***Table 3.2**: PLR and PIPR protocols used in preliminary testing*

Equipment	Protocol	Adaptation Details	Stimulus Details	Results Section(s)
NeurOptics PLR-3000	Positive Stimulus White Flash (PLR)	Ambient light conditions	1 s stimulus duration, 30 s ISI, 50 µW stimulus	3.3.1.1, 3.3.1.2
NeurOptics PLR-3000	Positive Stimulus White Flash (PLR)	5 minutes of dark adaptation	1 s stimulus duration, 30 s ISI, 50 µW stimulus	3.3.1.1
PyPlr	Positive Stimulus White Flash (PLR)	2 minutes of dark adaptation	1 s stimulus duration, 60 s ISI, 50 µW stimulus	3.3.2.1
PyPlr	Positive Stimulus White Flash (PLR)	2 minutes of dark adaptation	1 s stimulus duration, 90 s ISI, 50 µW stimulus	3.3.2.1, 3.3.3
PyPlr	Blue and Red Flash (PIPR)	2 minutes of dark adaptation	1 s stimulus duration, 60 s ISI, 800 lux stimulus. Alternating between blue (470 nm peak) and red (659 nm peak) light	3.3.2.2
PyPlr	Blue and Red Flash (PIPR)	2 minutes of dark adaptation	1 s stimulus duration, 90 s ISI, 800 lux stimulus. Alternating between blue (470 nm peak) and red (659 nm peak) light	3.3.2.2
PyPlr	Blue and Red Flash (PIPR)	2 minutes of dark adaptation	1 s stimulus duration, 120 s ISI, 800 lux stimulus. Alternating between blue (470 nm peak) and red (659 nm peak) light	3.3.2.2
NeurOptics PLR-3000	Positive Stimulus White Flash (PLR)	2 minutes of dark adaptation	1s stimulus duration, 60 s ISI, 50 µW stimulus	3.3.3

The NeurOptics and PyPlr spectral power distributions for this and subsequent chapters are shown in Appendix A.

3.2.1. Preliminary Neuroptics Testing

As the NeurOptics PLR-3000 has already been used in several peer-reviewed studies, this device was used to determine baseline values to optimize the protocols involving the use of white light to elicit the pupillary light response. The main factors to consider were the stimulus intensity and duration, surrounding light conditions, and whether to use a positive or negative flash protocol.

3.2.1.1. Ambient vs. Dark-Adapted Conditions

Data was acquired from four healthy subjects with no record of neurological disorders.

For the ambient light condition, subjects were all tested on the same day in a moderately lit testing room. For each subject, one measurement was taken in each eye, starting with the right eye. There was a 1 s baseline measurement, a 1 s flash of 50 μW white light, and 7 s of post-stimulus measurement, for a total measurement time of 9 s. The left eye was tested using the same protocol, within 20-30 seconds of the right eye measurement.

Two of the four subjects were included for further testing for the dark-adapted protocol, to compare the ambient light-adapted pupillary responses and the dark-adapted pupillary light responses of both eyes. After all ambient light condition measurements were completed, all the lights were turned off and left off for five minutes. The room has the windows blocked off with a full-length door, and so the room was close to pitch black aside from the light from the necessary electronic devices. After subjects were adjusted to the dark for five minutes, the PLR measurements were made in the same order as the ambient light condition

measurements were made in, with the same light stimulus – 1 s baseline measurement, a 1 s flash of 50 μW white light, and 7 s of post-stimulus measurement. The first measurement was done with the right eye, with the left eye measurement 30-50 seconds after.

3.2.2. Preliminary PyPlr Testing

The next pupillometry system to test was the PyPlr setup, which has benefits in that it can provide more control over the spectrum of light – this is contrary to the NeurOptics PLR-3000, which can only produce stimuli using set intensities of white light. Here, the primary aim was to test the white light stimuli using different interstimulus intervals, and to test a blue and red light protocol to elicit responses from maximal and minimal melanopsin sensitivity levels.

For each protocol, the PyPlr setup was one modified from that used in preliminary validation by Martin *et. al*. [18]. Before conducting any measurements, the system was calibrated using previous raw spectral measurements from an OceanOptics STS-VIS spectrometer (Ocean Insight Inc., Oxford, UK), as previously tested [18].

For the measurements, subjects sat in a chair and were instructed to place their chin on a chin rest, which were both height-adjusted to ensure maximal comfort. The chin rest was aligned with an integrating sphere, coated with Avian-B high reflectance paint to scatter light homogenously [18], which received light from the STLAB light source mounted and secured above an entry port [18]. The participant wore the Pupil Core eye-tracking headset. Both the STLAB light source and eye-tracking headset were connected to a computer running Pupil Capture and custom Python software. The hardware setup is shown in Figure 3.2.

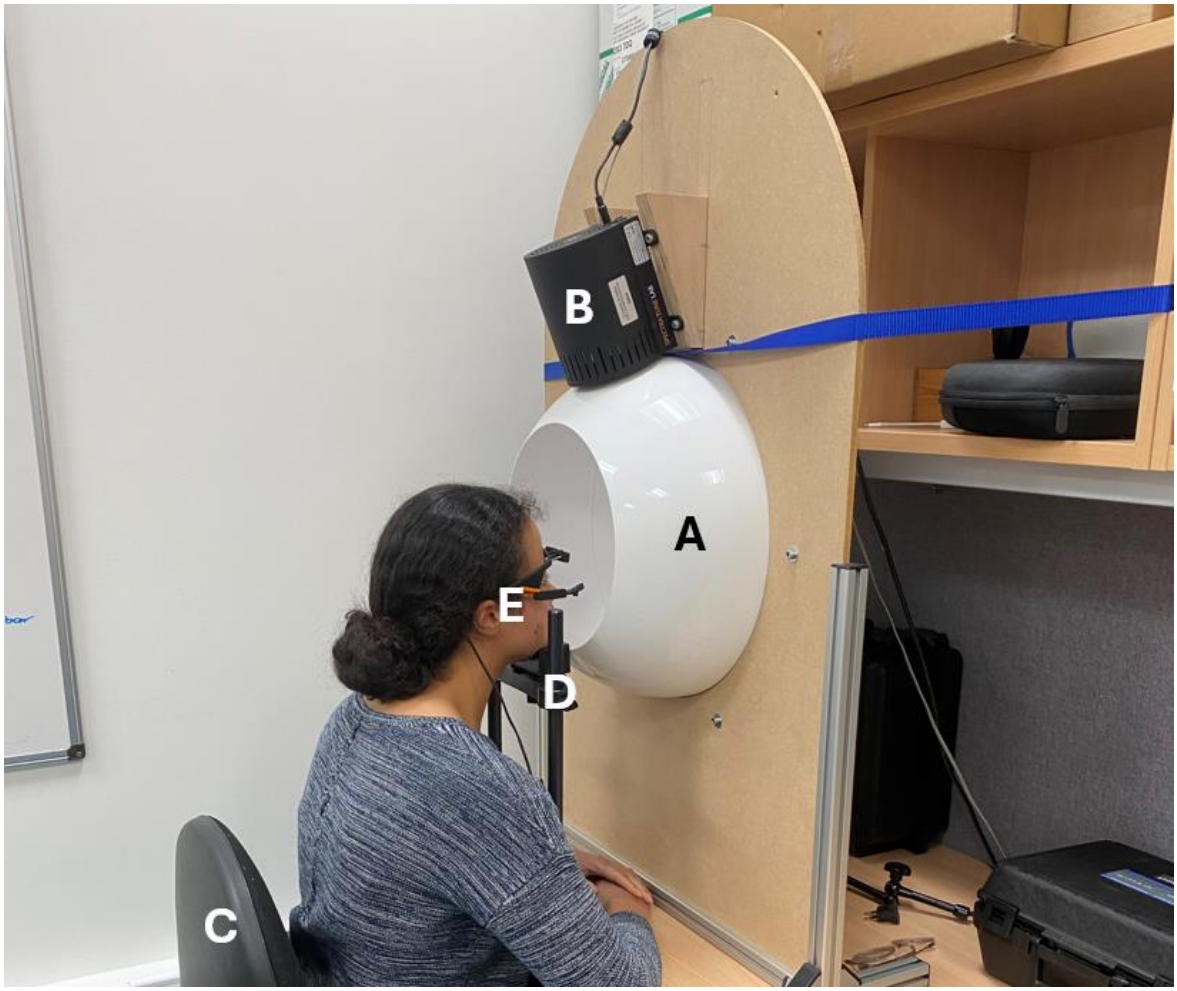

***Figure 3.2**: PyPlr hardware setup. This includes (A) the integrating sphere, stabilised using a wooden fixing plate, (B) STLAB light source mounted above an entry port to provide the light stimulus, (C) adjustable chair, (D) adjustable chin rest, and (E) Pupil Core eye-tracking headset, shown with the orange camera extenders. The light source and headset cables are both connected to a computer running Pupil Capture.*

From this setup, the light stimulus can be administered, and the pupil's response is measured. The Pupil Labs headset assigns a "confidence" value, ranging from 0 (no confidence) to 1 (full confidence), which enables data to be filtered if the subject blinks or if additional noise is introduced.

3.2.2.1. White light positive stimulus

The first tests done with the PyPlr system were to create a white flash stimulus, which, once refined, could be compared to the data collected from the NeurOptics PLR-3000. Two different time periods were tested: a 60 s ISI, and a 90 s ISI. Both

protocols were conducted in the same testing room as outlined in Section 3.2.1.1. One subject was used to compare the different time periods.

The 60 s ISI protocol was done first. For this protocol, all the lights were turned off and left off for two minutes. After the subject was adjusted to the dark for two minutes, the PLR measurement was conducted. This involved a 5 s baseline measurement, a 1 s flash of 50 μW white light, and 60 s of post-stimulus measurement before the next flash of white light. Three trials were conducted, and the baseline measurement for trials 2 and 3 were part of the 60 s post-stimulus measurement. Both eyes were flashed at the same time.

The 90 s ISI protocol was done next, on a different day (one week later) to ensure the participant had sufficient rest and avoided straining their eyes. For this protocol, the participant once again had two minutes in the dark testing room to become dark-adapted, after which the PLR measurement was conducted. This involved a 5 s baseline measurement, a 1 s flash of 50 μW white light, and 90 s of post-stimulus measurement before the next flash of white light. Once again, three trials were conducted, and the baseline measurement for trials 2 and 3 were part of the 90 s post-stimulus measurement. Both eyes were flashed at the same time.

Although the 60 s ISI protocol was done first, data from the first attempt of the 60 s ISI was too noisy to make any conclusions with, and so the subject was asked to return and measurements for the 60 s ISI were redone 9 days later from the original measurement, two days after the 90 s ISI protocol.

3.2.2.2. Blue and red light

The next tests were done to assess the blue and red light protocols to assess the post-illumination pupil response, originally tested by Martin *et. al.* in a modified set up [18]. Three different time periods were tested: a 60 s, 90 s, and a 120 s ISI. All protocols were conducted in the same testing room as outlined in Section 3.2.2.1, with the same subject used to compare the different time periods.

The 60 s ISI protocol was done first. For this protocol, all the lights were turned off and left off for two minutes. After the dark adaptation, the PIPR measurement was conducted. Each trial involved a 5 s baseline measurement, then a 1 s flash of blue light (470 nm peak) at 800 lux, followed by 60 s of post-stimulus measurement, a 1 s flash of red light (659 nm peak, matched for unweighted irradiance), followed again by 60 s of post-stimulus measurement. Three trials were conducted for a total of six flashes, and the baseline measurement for the last five flashes were part of the 60 s post-stimulus measurement. Both eyes were flashed at the same time.

After giving the subject a break, the 120 s ISI protocol was done next on the same day. This involved the same two-minute dark adaptation and the same sequence and intensity of lights, with a 120 s ISI instead of 60 s. Finally, after reviewing subject feedback, the 90 s ISI protocol was done nine days later – again, with the same two-minute dark adaptation and the same sequence and intensity of lights, with a 90 s ISI.

3.2.3. Comparing the NeurOptics with PyPlr

After deciding on appropriate ISIs for both the NeurOptics and PyPlr, data from 11 subjects were collected with the NeurOptics and PyPlr white flash protocols.

For the NeurOptics, this involved a similar procedure as outlined in Section 3.2.1.1, except with less dark adaptation time (2 minutes) and an interstimulus interval of 60 s. This was to ensure that sufficient recovery time was given with multiple trials to be performed. After subjects were adjusted to the dark for 2 minutes, the PLR measurements were taken with one eye at a time, starting with the right eye and alternating between eyes – 1 s baseline measurement, a 1 s flash of 50 μW white light, and 7 s of post-stimulus measurement. Three trials were administered for each eye.

With PyPlr, the same hardware setup described in Section 3.2.2 was used, and the same protocol defined in Section 3.2.2.1 for the 90 s ISI. The ISI was 90 s instead of 60 s used in the NeurOptics protocol, since both eyes were being tested at the same time rather than alternating and from previous testing, the 90 s ISI had worked best – this was a balance of allowing sufficient recovery while not taking too much additional time. Three trials were administered in total, capturing data from both eyes simultaneously.

3.3. Results

3.3.1. Preliminary NeurOptics Results

All subjects included for testing completed the protocols as expected.

3.3.1.1. Ambient light measurements

Table 3.3 shows the mean and standard deviation for the PLR metrics under the ambient light conditions across all subjects, and Figures 3.3 and 3.4 show the graphical representations of the PLR.

***Table 3.3**: Pupillary light response metrics for ambient light conditions*

	Initial Diameter (mm)	End Diameter (mm)	Latency (s)	Constriction Velocity (mm/s)	Max Constriction Velocity (mm/s)	Dilation Velocity (mm/s)	Time to 75% recovery (s)
Average	4.90 ± 0.68	2.88 ± 0.43	0.21 ± 0.04	-2.81 ± 0.50	-4.91 ± 0.77	1.34 ± 0.35	3.00 ± 1.51
Right Average	5.15 ± 0.71	2.95 ± 0.52	0.21 ± 0.04	-3.05 ± 0.38	-5.16 ± 0.96	1.32 ± 0.37	3.36 ± 1.81
Left Average	4.65 ± 0.65	2.80 ± 0.39	0.20 ± 0.03	-2.57 ± 0.52	-4.66 ± 0.55	1.35 ± 0.38	2.63 ± 1.31

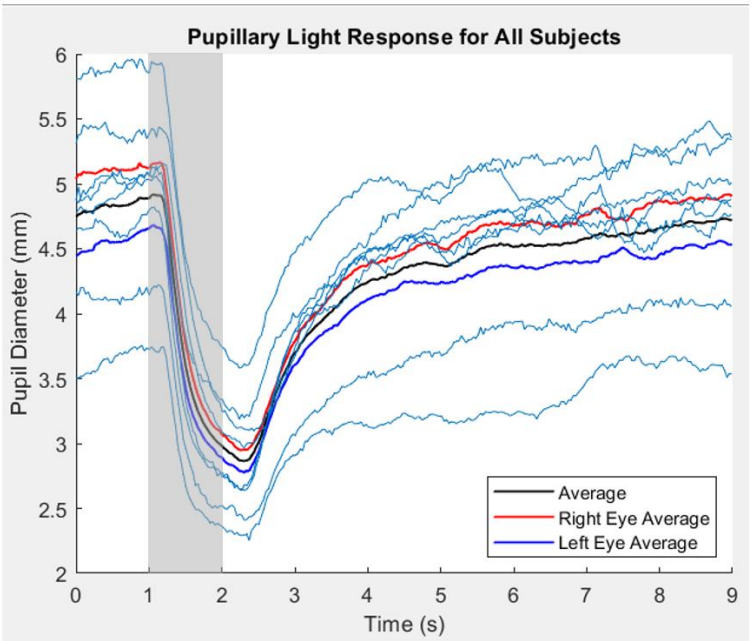

***Figure 3.3:** Pupillary light responses for all subjects in ambient light conditions, with the 1 s stimulus shown in the shaded area. Each individual subject is shown with a light blue trace.*

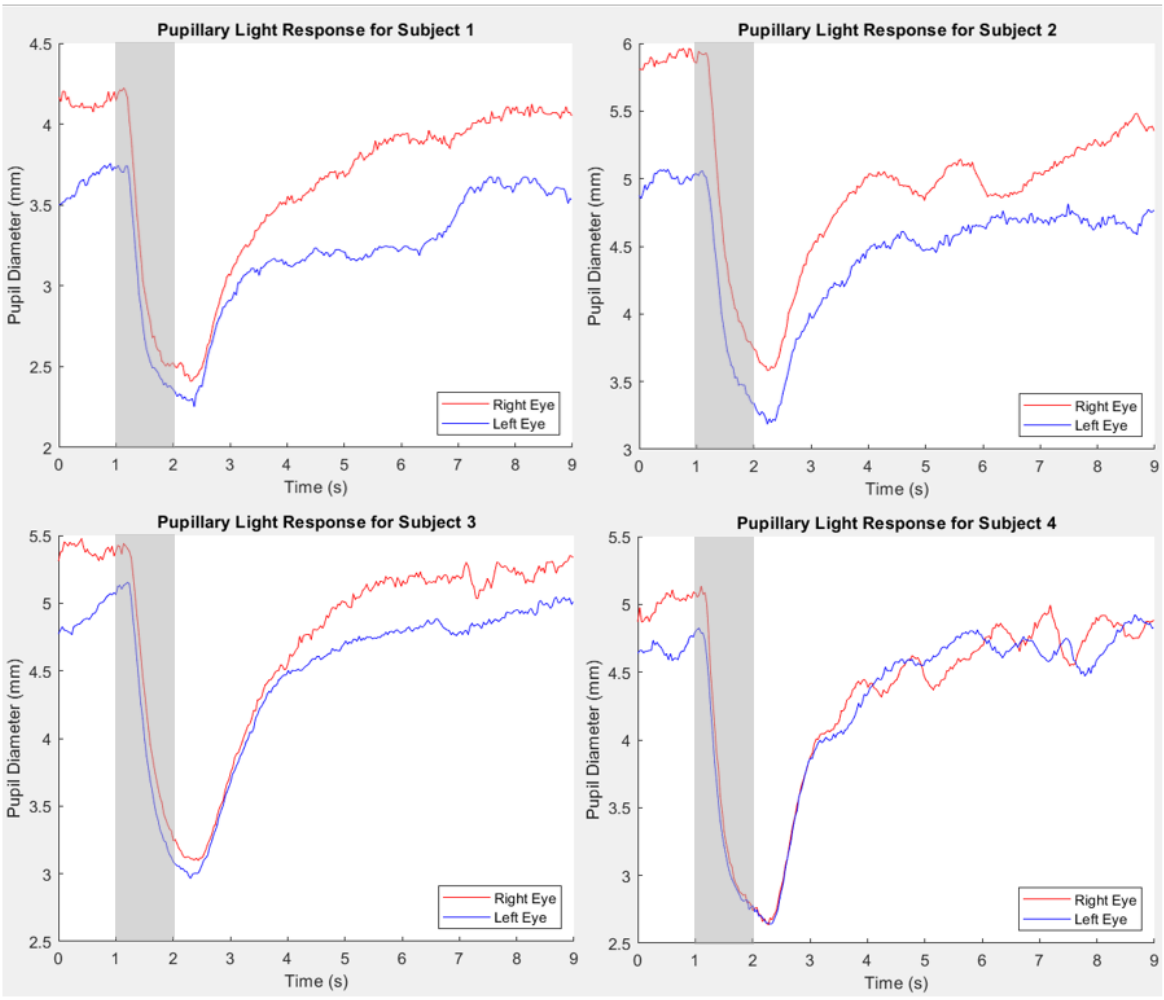

Figure 3.4: *Pupillary light responses for four individual subjects in ambient light conditions, with both the right and left pupil responses shown. The 1 s light stimulus is shown in the shaded area of all plots.*

3.3.1.2. Dark-adapted vs. light-adapted measurements

Table 3.4 shows the mean and standard deviation for the PLR metrics under the ambient light and dark-adapted conditions across all subjects, and Figures 3.5 and 3.6 show the graphical representations of the PLR under ambient light and dark-adapted conditions.

***Table 3.4**: Pupillary light response metrics for ambient light vs dark-adapted conditions*

	Initial Diameter (mm)	End Diameter (mm)	Latency (s)	Constriction Velocity (mm/s)	Max Constriction Velocity (mm/s)
Ambient Light Average	4.63 ± 0.43	2.93 ± 0.40	0.19 ± 0.02	-2.63 ± 0.56	-5.32 ± 1.85
Dark-adapted Average	7.70 ± 0.29	4.55 ± 0.65	0.28 ± 0.15	-3.05 ± 0.44	-5.54 ± 1.64

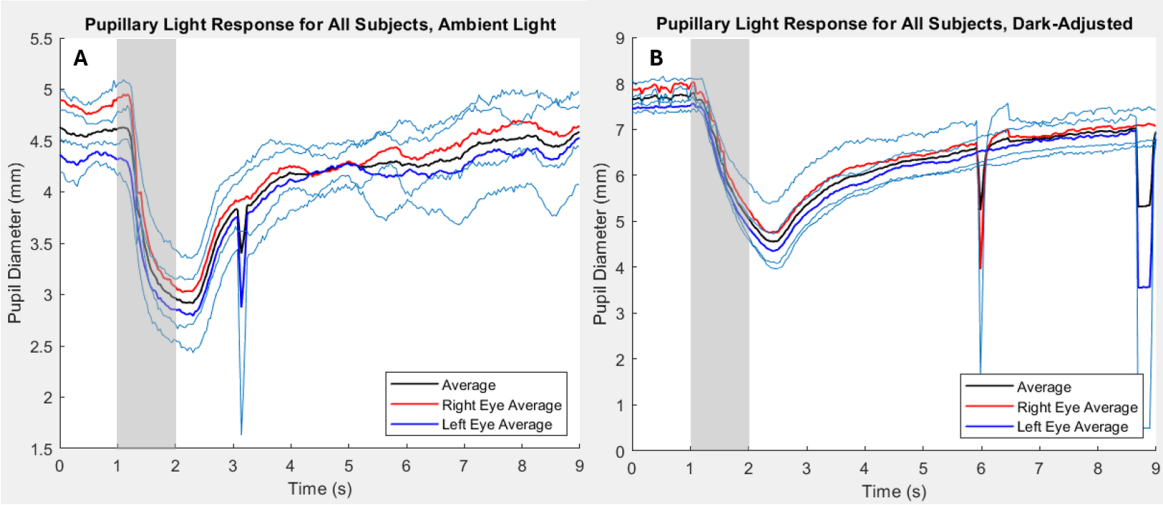

***Figure 3.5**: Pupillary light responses for all subjects in (A) ambient light conditions and (B) dark-adapted conditions, with the 1 s light stimulus shown in the shaded area of both plots. The sharp down spikes are due to blinking artefacts.*

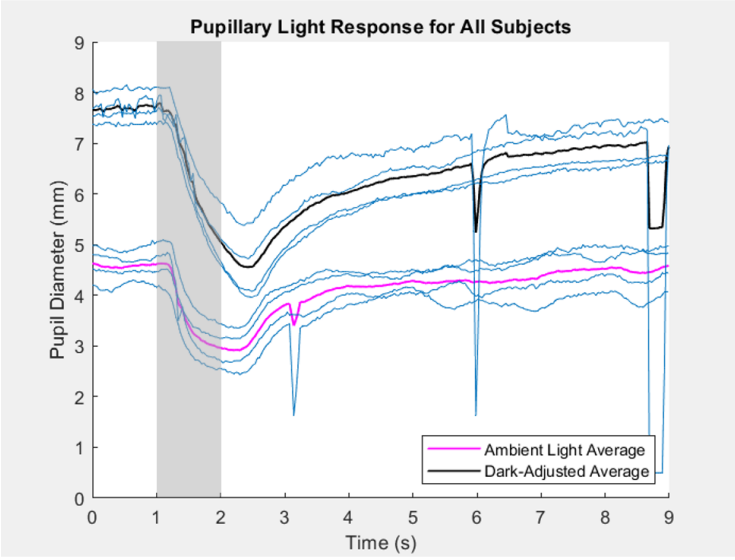

***Figure 3.6**: Comparison of pupillary light response in ambient light and dark-adapted conditions, with the 1 s light stimulus shown in the shaded area. The sharp down spikes are due to blinking artefacts.*

Because there were several blink artefacts that occurred while the pupil was dilating post-stimulus (seen as sharp down spikes in Figures 3.5 and 3.6), the dilation velocity and time to 75% recovery were not accurately measured and thus were not included in the table of average results for this test.

3.3.2. Preliminary PyPlr Results

3.3.2.1. White stimulus protocols

Figures 3.7 and 3.8 show the raw and processed data from the 60 s ISI protocol for the first and second attempts, and Figure 3.9 shows the raw and processed data from the 90 s ISI protocol for the same subject. For comparison, only the left eye is shown due to data completeness across all three attempts.

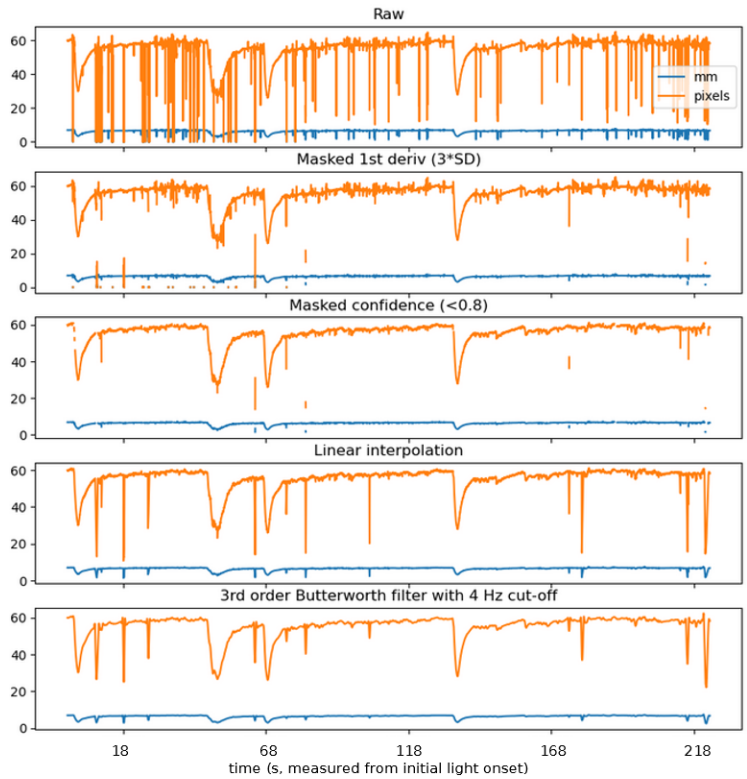

***Figure 3.7**: Subject data for the PyPlr white flash protocol with a 60 s interstimulus interval, first attempt, left eye. Initial light onset is at t = 0 s.*

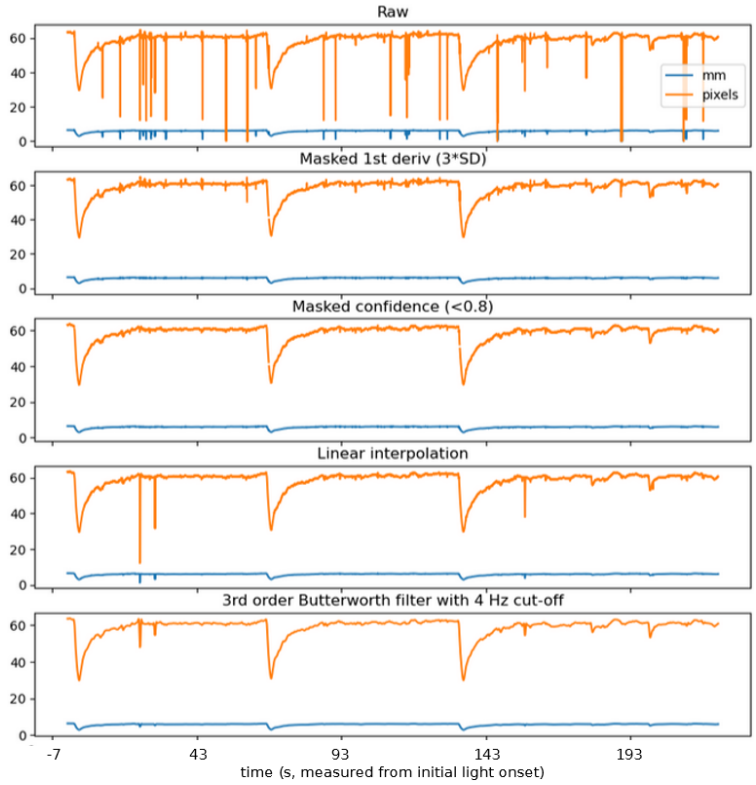

Figure 3.8: *Subject data for the PyPlr white flash protocol with a 60 s interstimulus interval, second attempt, left eye. Initial light onset is at t = 0 s.*

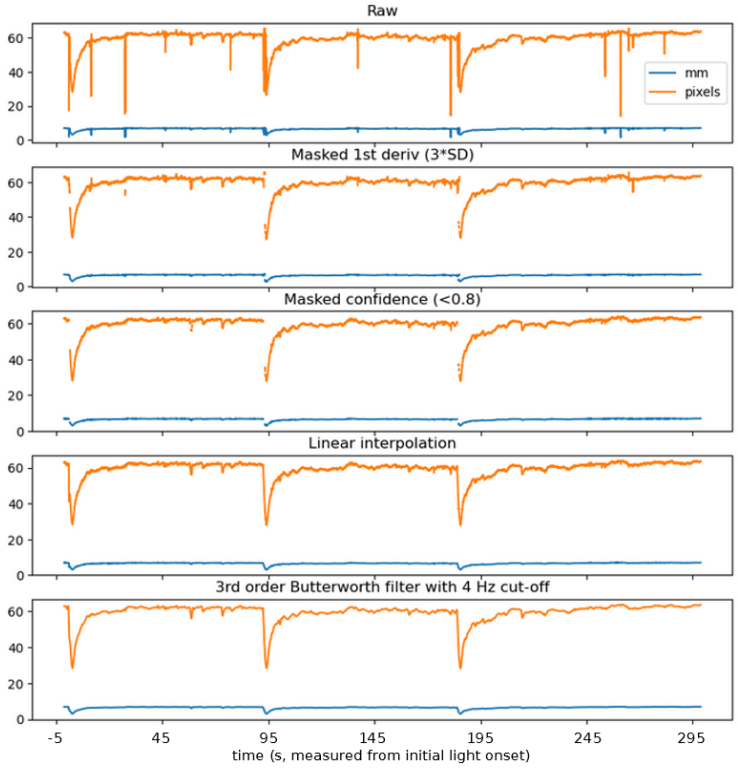

Figure 3.9: *Subject data for the PyPlr white flash protocol with a 90 s interstimulus interval, left eye. Initial light onset is at t = 0 s.*

After asking the subject their thoughts on the protocols, they suggested that 60 s was not sufficient recovery time for the stimuli, but that 90 s felt sufficient.

Full PLR parameters will be shown and compared in Section 3.3.

3.3.2.2. Blue and red stimulus protocols

Figures 3.10, 3.11, and 3.12 show the post-illumination pupil response to the 60 s, 90 s, and 120 s ISI. For better comparison, only the first 60 s of each response is shown, for the left eye of the subject. In the figures, the average response for the blue and red lights are shown in thicker and darker blue and red lines, with individual trials shown in lighter blue and red lines.

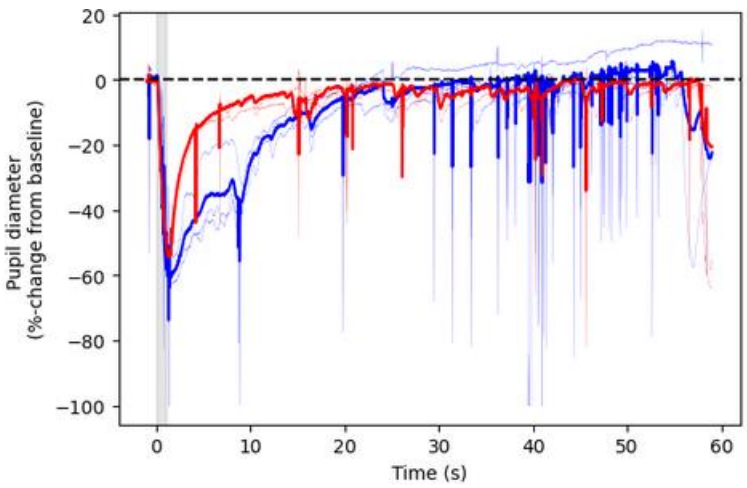

***Figure 3.10**: Subject data for the PyPlr blue and red flash protocol with a 60 s interstimulus interval, left eye. The 1 s light stimulus is shown in the shaded area of the plot.*

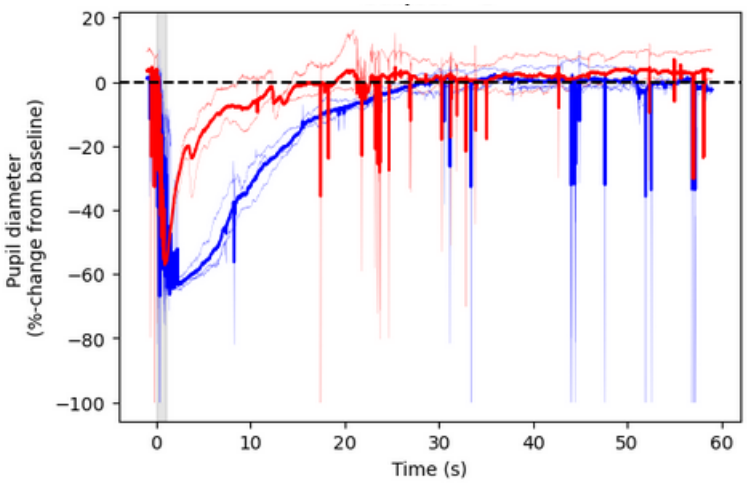

Figure 3.11: *Subject data for the PyPlr blue and red flash protocol with a 90 s interstimulus interval, left eye. The 1 s light stimulus is shown in the shaded area of the plot.*

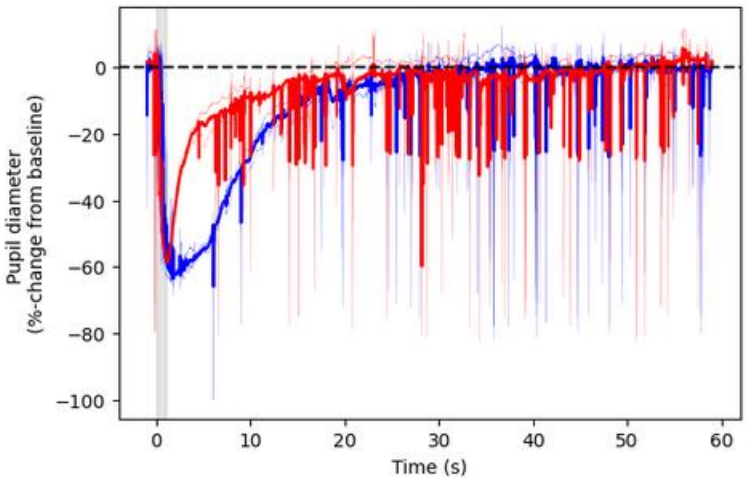

Figure 3.12: *Subject data for the PyPlr blue and red flash protocol with a 120 s interstimulus interval, left eye. The 1 s light stimulus is shown in the shaded area of the plot.*

Just as with the white flash protocols, the subject suggested that 60 s was not sufficient recovery time for the coloured stimuli in this protocol, but that 90 s felt sufficient and therefore they would prefer the 90 s ISI over the 60 s ISI. As the 90 s was sufficient for recovery, the 120 s ISI was not used in further experiments, and the 90 s ISI was used to allow for comparisons with the PyPlr white flash protocol.

3.3.3. Comparison of NeurOptics and PyPlr

All 11 subjects completed both the NeurOptics and PyPlr protocols with no issues.

Table 3.5 shows the PLR parameters from the NeurOptics and PyPlr, averaged across all 11 subjects for the white flash protocols.

***Table 3.5**: NeurOptics and PyPlr PLR Parameters*

PLR Metric	NeurOptics (Mean ± SD)	PyPlr (Mean ± SD)
Average Constriction Velocity (mm/s)	2.70 ± 0.55	1.56 ± 0.76
Maximum Constriction Velocity (mm/s)	5.31 ± 1.15	3.22 ± 1.40
Constriction Amplitude (mm)	2.66 ± 0.49	3.01 ± 0.43
Latency (s)	0.22 ± 0.02	0.30 ± 0.18
Dilation Velocity (mm/s)	1.20 ± 0.18	0.21 ± 0.18
Time to 75% Recovery (s)	4.35 ± 1.19	10.79 ± 3.87

Figure 3.13 shows the pupillary light response with the NeurOptics and PyPlr white flash protocols, in an example subject.

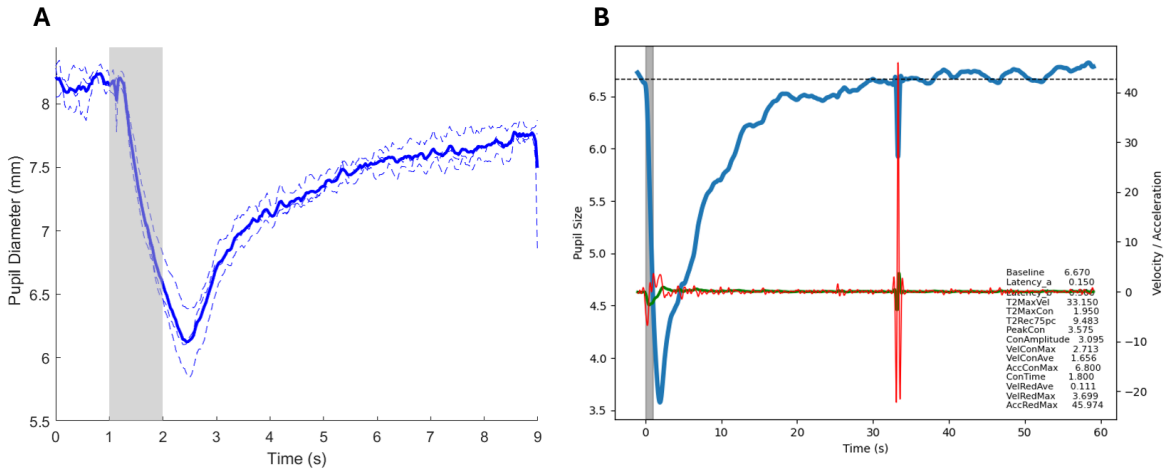

***Figure 3.13**: Pupillary Light Response in an example subject, using (A) the NeurOptics protocol and (B) PyPlr, with the 1 s light stimulus shown in the shaded area of both plots. In (A), the bolded blue trace represents the average response, with the dashed light blue traces representing individual trials. In (B), the thick blue line represents the average pupil response, with the red line representing the average acceleration of the response. Note that the large spike in the acceleration trace is due to noise.*

3.4. Discussion

Both the NeurOptics PLR-3000 and PyPlr pupillometry systems were able to assess the pupillary light response in various protocols, with strengths and weaknesses arising in specific testing conditions.

From the preliminary NeurOptics results, the right eye starting and ending diameters were higher than for the left eyes for all subjects. Additionally, the average and maximum constriction velocities were higher in the right eye than in the left, and the time to 75% recovery was longer in the right eye. These discrepancies are likely because for all participants, the right eye was assessed first, and that insufficient time was provided between measurements. It is known that changes in attention and arousal, including arousal caused from changes in an environment, can impact the pupillary response, and it is feasible that the initial pupillary stimuli may have impacted this. This is why in future work, a longer interstimulus interval was used to attempt to reduce these variations. However, even with a prolonged interstimulus interval, this also informs the need to keep testing the same eye first in a given PLR protocol – being consistent by either assessing the right or left eye first in future protocols will be essential to ensure that data can be reliably compared across subjects.

Blink artefacts were evident in the NeurOptics measurements, which unfortunately cannot always be avoided even when subjects are instructed to refrain from blinking. Since the post-stimulus time must be kept at a reasonable length to assess the dilation response, there may be more blink instances as participants must keep their

eyes open for at least 9 s, which emphasizes the need for multiple trials to ensure that data could be collected while accounting for the potential for blinks.

The magnitudes of the PLR metrics were higher in dark-adapted conditions when compared to ambient light conditions. This is as expected, as many studies that assess the PLR are dark-adapted with subjects waiting in the dark for 2-20 minutes. Additionally, the dark-adapted response can be more accurately compared to other studies, as the ambient light conditions are harder to control and vary immensely across days and locations. Because of this, the dark-adapted PLR will be used in future work to be able to have a higher range of values to identify potential differences.

The PyPlr system successfully assessed the pupillary light response with white light and blue and red light protocols, which elicit responses dominated by different photoreceptor classes. The 90 s ISI proved to be the preferred protocol from the subject's perspective and allowed for sufficient recovery post-stimulus to assess the responses in both the white flash and blue and red flash protocols. However, with both PyPlr protocols, there was more noise than in the NeurOptics protocols. Although the PyPlr system offers flexibility in the protocols and can, additionally, remove blink artefacts through the confidence metric that the Pupil Labs device offers, this noise can create unreliable results if the device is not properly calibrated.

When refining the protocols, there were additional tests that revealed problems with the PyPlr setup that needed to be addressed in the protocol setup. The first issue was that eyelashes often interfered significantly with the pupil detection capabilities. Dark eyelashes, especially those with mascara, could be mistaken for the pupil in

the pupil detection, which introduced significant uncertainty and noise in the measurements. This could generally be avoided by asking participants to remove any eye makeup, and for those with darker eyelashes, by setting thresholds for maximum and minimum pupil size to filter out data that is not physiologically agreeable, and by setting intensity thresholds for detecting the pupil.

Additionally, although the Pupil Labs headset does allow for minor adjustments of the infrared cameras, even with these adjustments the headset did not fit every subject, which meant that in several cases, measurements with minimal noise were not able to be done in both eyes simultaneously. In these cases, the headset was positioned so that one eye was prioritized based on the best calibration results and confidence measures from the Pupil Labs headset.

In comparing the NeurOptics and PyPlr white flash protocols, they had different capabilities and measured different values for the PLR parameters. The NeurOptics protocol had a smaller standard deviation in mean values for all PLR metrics except for the constriction amplitude, when compared to the PyPlr protocol, likely attributed to the additional measurement noise in the PyPlr protocol. The NeurOptics protocol was limited in its ability to calculate time to 75% recovery, due to only measuring 7 s post-stimulus, whereas the PyPlr protocol was able to capture the entire recovery period, providing valuable insights to the full recovery. However, the longer post-stimulus measurement period for the PyPlr protocol also meant that the average dilation velocity was much smaller than in the NeurOptics protocol. Since the NeurOptics PLR-3000 has been used and validated in several peer-reviewed studies and is widely used in research and clinical practice, the NeurOptics white flash

protocol will be the protocol used in future work, to reduce noise and to allow for further comparisons with other studies. However, the PyPlr system's blue and red light protocol will also be used, to evaluate the post-illumination pupil response.

In future chapters, the NeurOptics and PyPlr protocols will be used, to determine which parameters and, which methods, display a relationship with cerebrovascular reactivity. To do this, a group of approximately 20 will be recruited to perform the measurements. For each subject, the PLR will be assessed using both protocols – the white flash using NeurOptics, and blue and red flash using PyPlr. Measurements will be taken from both eyes, with enough time between measurements for the pupil to return to the baseline pupil diameter.

The NeurOptics PLR-3000 can also perform a negative stimulus. The negative stimulus has not been as widely adopted as the positive stimulus for assessing the pupillary light response, however it can still give important information about the dilation parameters. Given the ease of operation of the NeurOptics, there is potential to implement this protocol to obtain a wider variety of parameters, to potentially further bias the dilation parameters while having a smaller relative contribution from the rods. This stimulus will be further explored in future chapters.

3.5. Conclusion

This chapter presented preliminary testing data from two pupillometry systems used to assess the human pupillary light response. Both the NeurOptics PLR-3000 and PyPlr system were able to assess the pupillary light response using various protocols, designed to elicit responses dominated by different photoreceptors. For a simple white flash protocol, the NeurOptics device presents the most consistent and

accurate data, while the PyPlr system offers increased flexibility to evaluate the post-illumination pupil response to blue and red light. Both pupillometers will be used in future work to compare the PLR and PIPR with other metrics, including those characterising brain vascular health.

Chapter 4: Characterising cerebrovascular reactivity and the pupillary light response – a comparative study

The aim of this chapter is to investigate smooth muscle function in both the eye and the brain, through an assessment of the pupillary light response and cerebrovascular reactivity, respectively. This chapter presents a pilot study that establishes a baseline relationship between parameters of the pupillary light response and cerebrovascular reactivity, using standard protocols in healthy adults. This chapter presents the first study comparing the PLR to CVR, and thus presents an important first step in defining the PLR-CVR relationship to evaluate cerebral blood flow and pupillary dynamics.

As this is the first study that has investigated the relationship between cerebral blood flow and pupil dynamics, this provides an important baseline that will enable future comparisons between the PLR and CVR in at-risk groups for dementia with covariate analyses, as well as with novel protocols for PLR and CVR assessment. This work will enable further investigation into impairments in smooth muscle function that may manifest in both CVR and PLR.

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: These authors have contributed equally to this work and share first authorship

Author contributions: S. Sparks contributed to the pupillometry study concept and design, acquisition, analysis and interpretation, writing of the manuscript. G. Hayes contributed to the CVR study concept and design, acquisition, analysis and interpretation, writing of the manuscript. J. Pinto contributed to ethics approvals, data acquisition, and critical revision of the manuscript. D.P. Bulte developed the original study concept and design, critical revision of the manuscript, and study supervision. All authors read and approved the final manuscript.

4.1. Abstract

Introduction: Smooth muscle is integral to multiple autonomic systems, including cerebrovascular dynamics through vascular smooth muscle cells and in ocular muscle dynamics, by regulating pupil size. In the brain, smooth muscle function plays a role in cerebrovascular reactivity (CVR) that describes changes in blood vessel calibre in response to vasoactive stimuli. Similarly, pupil size regulation can be measured using the pupillary light response (PLR), the pupil's reaction to changes in light levels. The primary aim of this study was to explore the interplay between cerebral blood flow and pupil dynamics, evaluated using CVR and PLR, respectively.

Methods: A total of 20 healthy adults took part in a CVR gas stimulus protocol and a light and dark flash PLR protocol. CVR was calculated as the blood flow velocity change in the middle cerebral artery, measured using transcranial Doppler ultrasound in response to a 5% increase in CO_2. Multiple PLR metrics were evaluated with a clinical pupillometer.

Results: CVR and PLR metrics were all within the expected physiological ranges for healthy adults. Nine different PLR metrics, assessed through the light and dark flash protocols, were compared against CVR. A significant negative relationship was observed between the latency of the PLR in the dark flash protocol and CVR. No statistically significant relationships were found between CVR and other PLR metrics.

Conclusion: This is the first study to investigate the relationship between cerebral blood flow and pupil dynamics. A significant relationship between dark flash latency and CVR was observed. Future work includes evaluating these relationships using more robust CVR and PLR measurement techniques in a larger, more diverse cohort. Notably, more research is warranted into the PLR using a dark flash protocol and its connection to cerebrovascular function.

4.2. Introduction

Cerebrovascular dynamics are crucial for the maintenance of adequate cerebral blood flow (CBF) to the brain and can be quantified using a metric known as cerebrovascular reactivity (CVR). CVR describes the intrinsic ability for cerebral blood vessels to dilate and constrict in response to vasoactive stimuli, a phenomenon that is largely mediated by vascular smooth muscle cells (VSMCs) that surround arteries and arterioles [247], [248].

CVR can be measured by varying the arterial partial pressure of CO_2 ($PaCO_2$), inducing either hypercapnia (increased $PaCO_2$) or hypocapnia (decreased $PaCO_2$) through stimuli such as voluntary breathing tasks, gas protocols, or acetazolamide injection [249], [250], [251]. The concomitant CBF changes can be measured non-

invasively using an appropriate imaging modality such as magnetic resonance (MR) imaging or transcranial Doppler ultrasound (TCD). While MR provides CVR measures with relatively high spatial resolution including brain micro-vasculature [252], TCD is a simpler, more widely available and cost-effective alternative that measures blood velocity in single major arteries [253], [254]. Measurements of CVR are emerging in clinical use to assess cerebrovascular function including in Alzheimer's disease and dementia [255], [256], [257], [258], [259], carotid artery stenosis [260], [261], stroke [262], congestive heart failure [263], hypertension [264], [265].

Smooth muscle can also be found outside of the brain, such as in the iris in the form of sphincter and dilator muscles to control the size of the pupil [266]. These muscles can be easily assessed using the pupillary light response (PLR, also called the pupil flash reflex). The PLR characterises pupillary size changes to different light conditions [267]. These changes are mainly controlled by opposing branches of the autonomic nervous system: whilst the parasympathetic nervous system controls the constriction facilitated by the sphincter muscles of the iris, the sympathetic nervous system controls the dilation facilitated by the dilator muscles of the iris [268], [269], [270]. In response to a light stimulus, the PLR can be categorised into four dynamic phases: response latency, maximum constriction, pupil escape, and recovery [271]. Various parameters of the PLR can be extracted from these four phases for further assessment, depending on the application.

The PLR has been used in clinical and research settings as a diagnostic tool for several mental and physical health problems, including acute and traumatic brain injury [272], [273], [274], depression [275], [276], [277], [278], diabetes [279], [280],

[281], [282], and increased intracranial pressure and intracranial hypertension [283], [284], [285], [286], [287]. Changes in the PLR have also been reported in both preclinical and clinical Alzheimer's disease cases [270], [288], [289], [290], as well as in those identified to have increased risk of developing neurodegenerative disorders [291].

Given that both of these measures appear to be related to a variety of factors including smooth muscle dynamics and function, and additionally show overlapping changes in several pathologies, it is important to investigate their association to better understand pathological mechanisms and their identification. Therefore, this pilot study aims to explore the relationship between the PLR and CVR in healthy adults with no history of cerebrovascular or eye disorders as a means of assessing the interplay between dynamics in the brain and in the pupil.

4.3. Materials and Methods

4.3.1. Subjects

We acquired data from twenty healthy subjects with no record of neurological disorders (9F, age range 23-68 years, with a mean of 33.5 ± 11.5 years at the time of acquisition). Inclusion criteria consisted of having no diagnosed cognitive impairment, psychiatric conditions, diabetes, high blood pressure, respiratory, or cardiac health issues. Participants with corrective prescription glasses did take part in the study, but none who had known vision loss and none who had undergone eye or brain surgery. They were also instructed to refrain from consuming caffeinated drinks for two hours before the session. All participants provided informed written consent before each session, and the study was approved by the Medical Sciences

Interdivisional Research Ethics Committee (MS IDREC) of the University of Oxford's Central University Research Ethics Committee (CUREC).

4.3.2. Data Acquisition

Data acquired in this study included cerebral blood velocities using TCD and a respiratory gas stimulus, and pupil dynamics using pupillometry with light stimuli. For all participants, the sequence of protocols involved the completion of the TCD and gas stimulus first, followed by the dark adaptation and pupillometry protocols, with at least 10 minutes of time between protocols to change equipment and transfer setups.

4.3.2.1. Transcranial Doppler Ultrasound and Gas Stimulus

A 2 MHz pulsed transcranial Doppler ultrasound system (7760EN Doppler-BoxX Digital, Compumedics DWL) was used to measure cerebral blood velocities in the middle cerebral artery (MCA). A transmission gel was applied to the transtemporal window of the volunteer and the TCD probe was placed over the gel and secured using an adjustable headset. The location and angle of the probe was changed until a steady blood flow velocity with good signal-to-noise ratio was achieved.

CO_2 and O_2 levels in respired air were sampled using a thin nasal cannula placed into both nostrils and an infrared gas analyser (ML206, ADInstruments). The CO_2, O_2, and TCD signals were recorded using a PowerLab 8/35, 8 Channel recorder (PL3508 ADInstruments) and accompanying LabChart Software.

For the gas stimuli, a custom gas delivery system was used to carry out the procedure and accurately monitor physiological parameters throughout it. This

system was built in-house at the University of Oxford [292]. It consisted of a disposable non-rebreathing anaesthetic face mask with a Laerdal bag placed over the participant's nose and mouth, secured using a head strap. Holes on either side of the mask were covered by unidirectional silicon membranes to allow exhaled air to escape the mask while being sealed during inhalation. A medical-grade respiratory filter was placed at the junction of the disposable circuit and the permanent fixtures to prevent cross-contamination. On the permanent side of the filter, a short length of tubing led to a parallel Y-pieces where respiratory gas mixtures could be delivered one at a time.

Two different levels of inspired gases (medical air and air with 5% CO_2) were delivered to the face mask at a rate of 15 L/min through unidirectional tubing. The gas cylinders, each fitted with a pressure regulator and flow metres, were operated by hand, following a predefined protocol.

The gas stimulus protocol consisted of a period of baseline measurements of blood flow velocity while the subject breathed normally on medical air for 3 minutes. After this period, the gas was switched from synthetic medical air (21% O_2 / 79% N_2) to a mixture of 5% CO_2 balance air (BOC Group, Linde, Surrey, UK) for another 3 minutes and the subject was instructed to continue breathing normally. Finally, the gas was switched back to medical air and another baseline measurement was taken for 2 minutes.

4.3.2.2. Pupillometry and Light Stimuli

The NeurOptics PLR-3000 hand-held pupillometer was used to measure the pupillary light response (NeurOptics, Irvine, CA). This hand-held pupillometer uses an infrared

camera to capture and measure the pupil size, is automated and monocular, and is widely used in clinical practice and research settings.

There were two protocols used to assess the pupillary light response. Before both protocols, subjects had 2 minutes of adaptation in a dark, quiet testing room, and throughout the pupillometry testing the subject was sat in a chair. Each protocol was done using the NeurOptics PLR-3000 device on one eye at a time.

For each subject, six measurements were performed on each eye, alternating eyes between each trial, starting with the right eye. The first three measurements on each eye, the light flash protocol, were with the positive step-input stimulus, which had a 1 second baseline measurement, a 1 second flash of 50 μW white light, and 7 seconds of post-stimulus measurement, with a 1-minute interstimulus interval. The last three measurements on each eye, the dark flash protocol, were with the negative step-input stimulus, which had a 1 second baseline measurement with the 50 μW light on, a 1 second dark flash with the light off, and a 7 second measurement with the light back on, also with a 1-minute interstimulus interval. These two protocols were matched to be the opposites of each other for comparison of the positive and negative pulses and the responses they evoked in subjects. The spectral information for the stimulus is available in Figure A.1 of Appendix A.

During the measurements, subjects were instructed to keep their eyes wide open and to avoid blinking, and to hold a constant gaze position. The pupillometer was held at a right angle to the subject's line of sight. All measurements were taken between 09:00 and 16:00 to avoid interference from circadian rhythms.

4.3.3. Data Analysis

4.3.3.1. Cerebrovascular Reactivity Analysis

The CO_2, O_2, and TCD, and time courses were exported at a time resolution of 200 Hz and processed using custom scripts in Python 3.10.8. The CO_2 and O_2 signals were converted from percent to mmHg using a conversion factor based on the midday pressure reading on the day of each acquisition in Oxford, UK [293]. The raw Doppler-BoxX TCD outputs were converted to cm/s using a calibration factor of 202.1 cm/s/V based on the DWL application software and values below 14 cm/s were removed since they corresponded to the bottoming out of the signal.

Two minutes of near-steady state data were extracted from each of the baseline and 5% CO_2 periods, starting at least 30 seconds after a transition. The end-tidal CO_2 ($P_{ET}CO_2$) peaks were automatically individuated using tools from the SciPy package [294] to be used as a surrogate for arterial $PaCO_2$ [295]. The mean $P_{ET}CO_2$, $P_{ET}O_2$, and TCD blood flow velocity were taken within each segment. The CVR was calculated by dividing the relative change in measured blood flow velocity by the change in the mean $P_{ET}CO_2$ between the segments as shown in Equation 4.1, where $MCAv_{5CO2}$ and $MCAv_{baseline}$ are the mean blood flow velocities during the 5% CO_2 gas and baseline medical air segments respectively, and the $P_{ET}CO_{2\ 5CO2}$ and $P_{ET}CO_{2\ baseline}$ are the mean end-tidal CO_2 values within each segment.

$$\boldsymbol{CVR}(\%/\boldsymbol{mmHg}) = \frac{\frac{\overline{MCA_{v}}_{5CO_2} - \overline{MCA_{v}}_{baseline}}{\overline{MCA_{v}}_{baseline}}}{P_{ET}CO_{2\ 5CO_2} - P_{ET}CO_{2\ baseline}} \cdot \mathbf{100} \qquad \textit{(eq. 4.1)}$$

4.3.3.2. Pupillometry Analysis

The time course data from the pupillometry experiments were extracted directly from the NeurOptics PLR-3000 pupillometer in a CSV file format and processed using custom scripts in MATLAB.

The NeurOptics pupillometer automatically calculates several metrics: initial and end pupil diameters, latency, average and maximum constriction velocity, dilation velocity, and time to 75% recovery for each 9 second measurement. All values were averaged across all trials for each participant.

Due to the nature of the PLR protocols, constriction parameters, dominated by the sphincter muscles and parasympathetic nervous system, were only assessed in the light flash protocol, as the dark flash protocol's constriction amplitude was significantly smaller than that of the light flash protocol where the pupil starts at a larger, dark-adapted diameter. Dilation parameters, however, were assessed in both the light and dark flash protocols, and have contributions from the dilator muscles and sympathetic nervous system as well as the sphincter muscles and parasympathetic nervous system.

For the light flash protocol, the key parameters that were assessed were (a) the average constriction velocity, (b) the maximum constriction velocity, (c) the constriction amplitude, (d) the dilation velocity, (e) the time to 75% recovery, and (f) the latency of the response. For the dark flash protocol, the key parameters assessed were (g) the dilation velocity, (h) the dilation amplitude, and (i) the latency of the response. All these parameters are visually depicted in Figure 4.1.

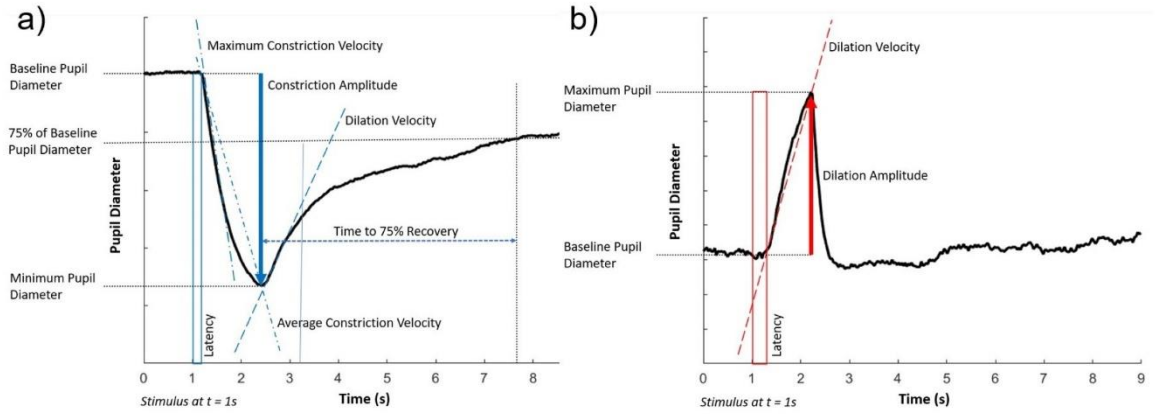

Figure 4.1: *Key components of the pupillary light response to a) the light flash protocol (positive stimulus) and b) the dark flash protocol (negative stimulus). Each stimulus starts at 1s and lasts for 1 second. Note that the latency in the dark flash protocol (shown in a red box) is longer than in the light flash protocol (shown in a blue box).*

4.3.3.3. Comparative Analysis

To identify any statistically significant relationships between the PLR and CVR, we performed linear regression analysis (significance level $p < 0.05$, uncorrected).

4.4. Results

Data from 18 of the 20 subjects were included for analysis. One of the subjects was excluded due to a noisy TCD signal which was likely the result of the probe moving out of alignment with the MCA during the gas protocol. The other participant was excluded due to recent history of smoking, as this could have been a confounding factor to the results.

4.4.1. Cerebrovascular Reactivity Results

The TCD derived blood flow velocity, CO_2, and O_2 traces for a representative subject during the protocol are shown in Figure 4.2 where the baseline and 5% CO_2 gas stimulus periods are both highlighted.

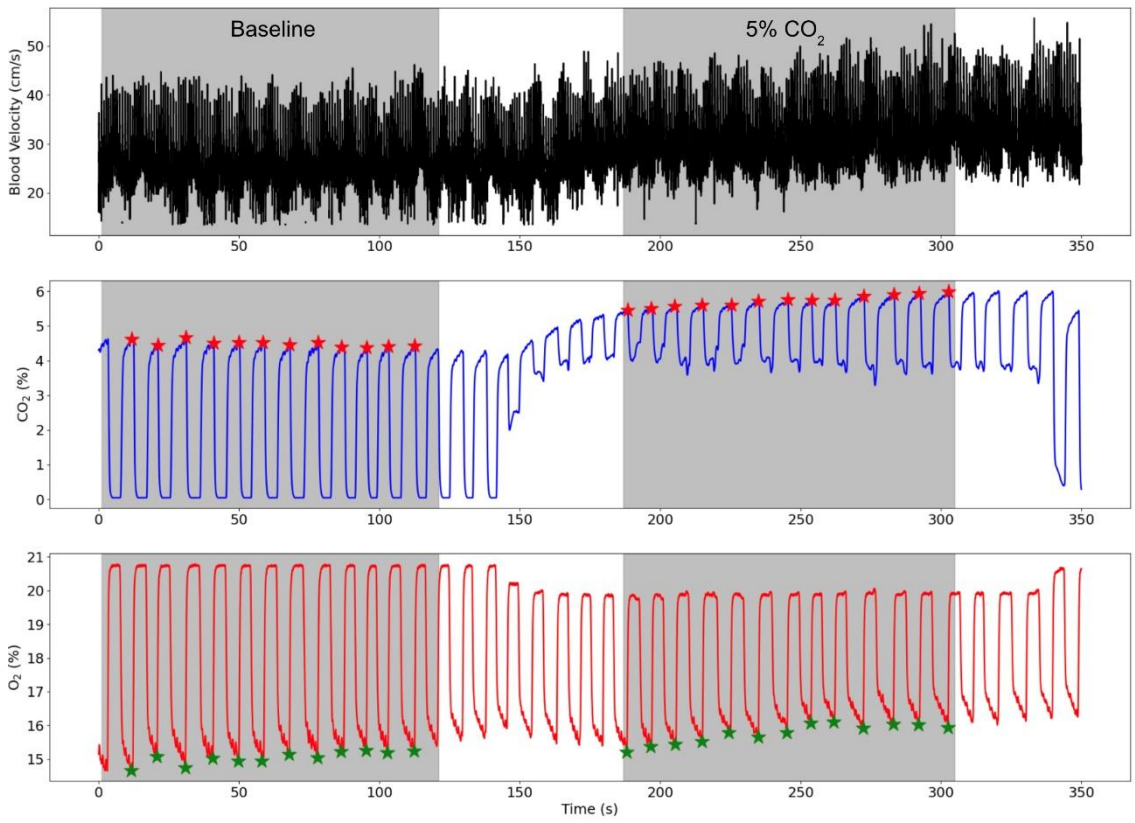

***Figure 4.2**: TCD blood flow velocity (cm/s), CO_2 (%), and O_2 (%) traces for a representative subject while the subject breathed medical air (baseline) and air with 5% CO_2 gas. The baseline and 5% CO_2 periods are shaded in grey and the end-tidal points for the CO_2 and O_2 traces are illustrated by red and green stars respectively.*

$P_{ET}CO_2$ significantly increased from baseline with a mean $P_{ET}CO_2$ difference between the 5% CO_2 hypercapnia period and baseline periods across subjects of 10.01 ± 2.05 mmHg (t-statistic = 9.17, $p << 0.01$). Similarly, MCA blood flow velocity increased with hypercapnia from baseline with a mean difference across subjects of 9.43 ± 3.24 cm/s (t-statistic = 3.83, $p << 0.01$). Breathing rates, end tidal points, mean blood flow velocities varied between subjects, but all were within normal and expected ranges [253], [263], [296], [297]. CVR was calculated using Equation 1 (relative change in MCA velocity compared to the change in $P_{ET}CO_2$), yielding an average CVR value of 2.90 ± 0.56 %/mmHg, across all subjects.

Minimal differences were observed in breathing rate and heart rate between the baseline period and the 5% CO_2 period. The mean and standard deviation of the breathing rate and heart rate for each period across all 18 subjects are presented in Table 4.1.

***Table 4.1**: Mean and standard deviation of the breathing rate in breaths per minute ($b_r$pm) and heart rate in beats per minute (bpm) of the participants during the baseline period and during the 5% CO_2 period.*

Metric	**Mean ± Standard Deviation**
Baseline Breathing Rate ($b_r$pm)	11.9 ± 5.5
5% CO2 Breathing Rate ($b_r$pm)	12.0 ± 5.0
Baseline Heart Rate (bpm)	69.1 ± 10.3
5% CO2 Heart Rate (bpm)	71.9 ± 6.7

4.4.2. Pupillometry Results

The pupillary light and dark responses for the same representative subject in their right eye is shown in Figure 4.3, where the mean response across all three trials in the right eye is highlighted.

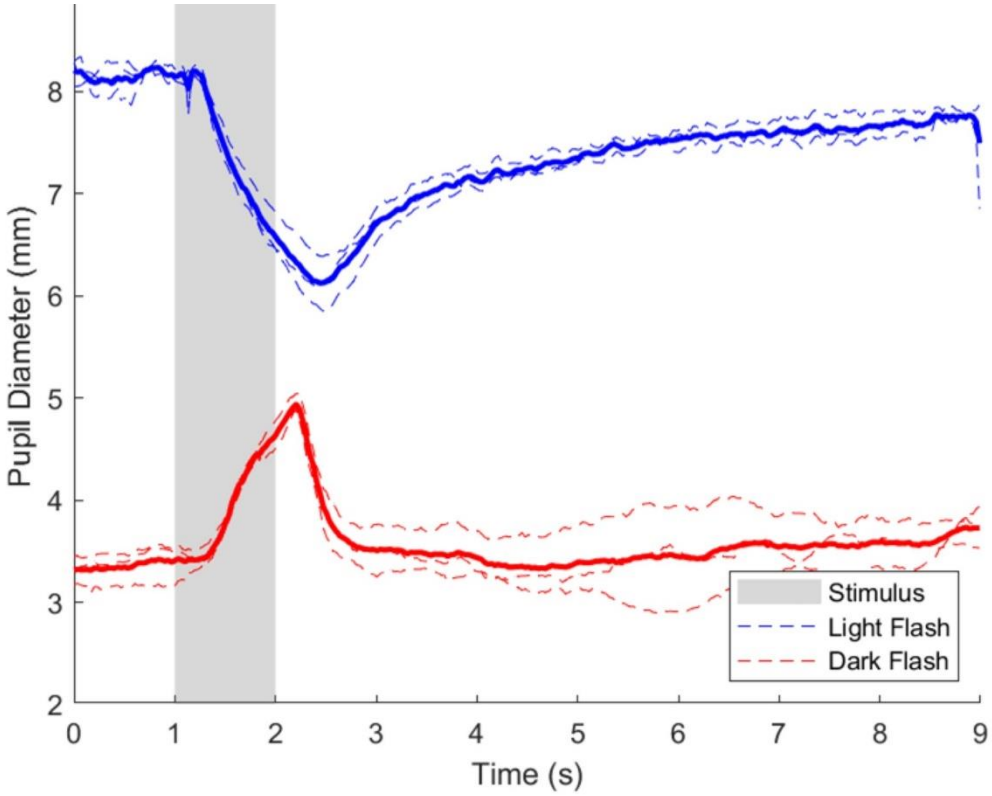

***Figure 4.3**: Pupillary light and dark flash response for the right eye of a representative subject. Three trials were performed in the right eye for both the light and dark flash protocols, which are shown on the plot in dashed blue and red lines, respectively. The average response of the light and dark flash protocols in the right eye across trials is shown in a thicker blue and red line, respectively. The stimulus for both protocols started at t = 1 s and ended at t = 2 s, and is shown in a shaded area on the plot.*

Data from both eyes were collected to ensure that any inconsistencies among subject eyes were noted. However, for the analysis, only the right eye was included for further analysis due to the more complete data among all included subjects. This was also to ensure that averaging across both eyes did not introduce any artefacts.

Figure 4.3 shows minor differences among individual trials, but the overall pupillary light response characteristics in the right eye were as expected and were comparable to previous studies [298], [299]. The interstimulus interval selected was sufficient for the pupil diameter to return to baseline before subsequent trials. Data from all three trials was averaged to account for minor variations due to hippus and

other minor physiological variations that can be expected in assessing pupillary dynamics [300].

4.4.3. Comparison Results

The constriction parameters of the light flash protocol compared to CVR are shown in Figure 4.4. The dilation parameters of both the light and dark flash protocol compared to CVR are shown in Figure 4.5. Finally, the latency in both the light and dark flash protocol compared to CVR is shown in Figure 4.6.

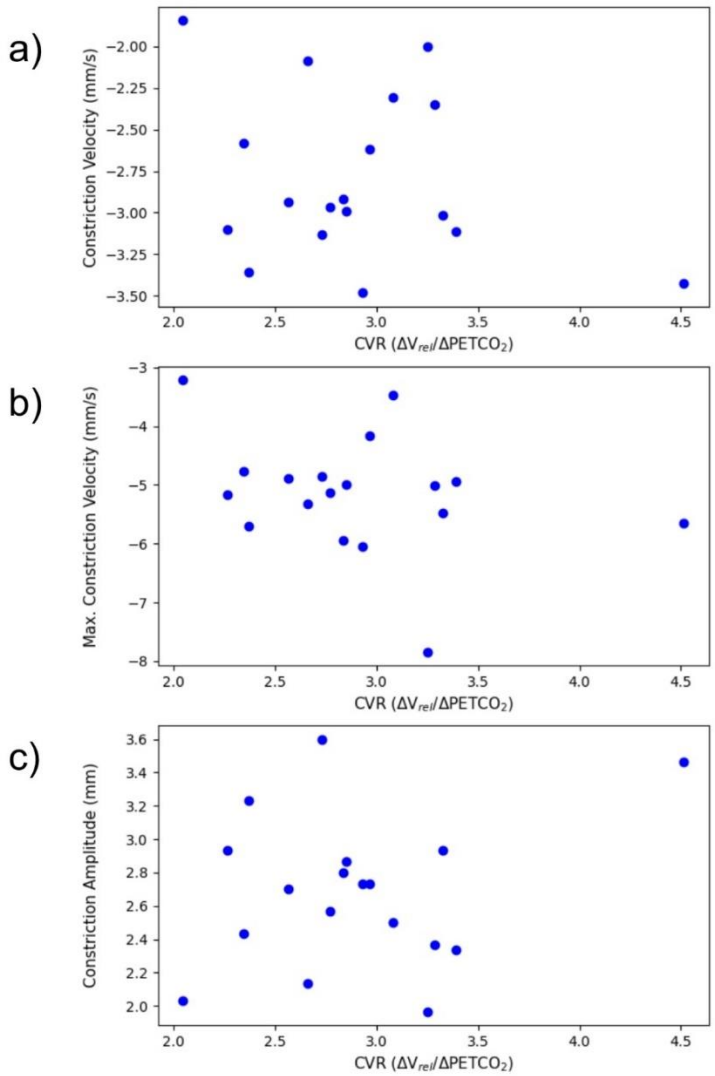

***Figure 4.4**: Constriction parameters of the light flash protocol, plotted against CVR. This includes a) the average constriction velocity (p = 0.307), b) the maximum constriction velocity (p = 0.201), and c) the constriction amplitude (p = 0.349), all from the light flash protocol compared to the CVR.*

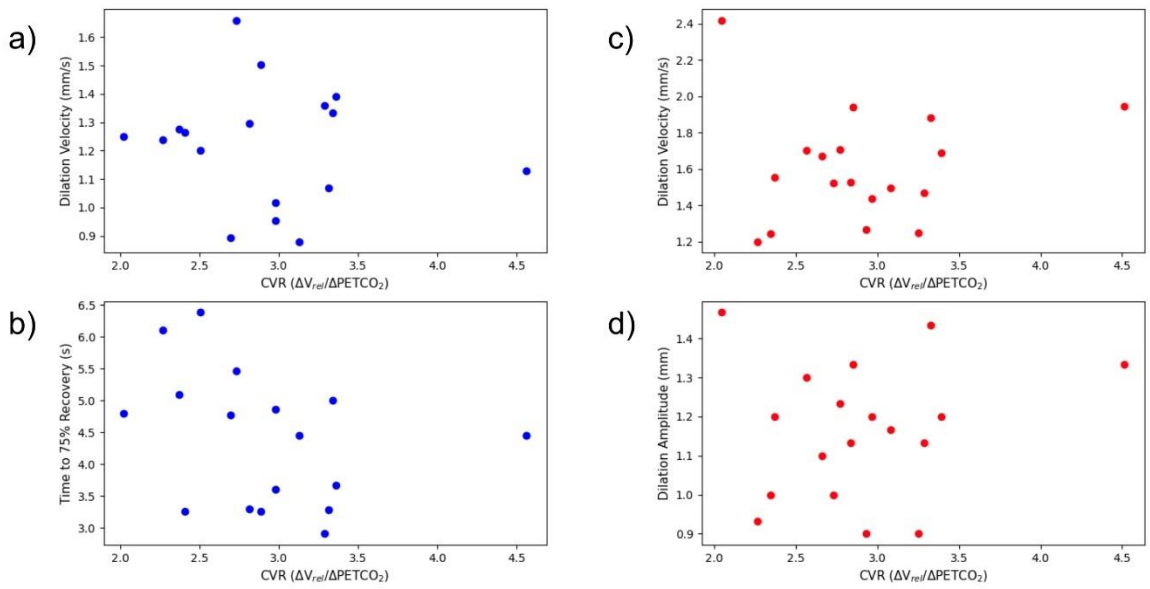

***Figure 4.5**: Dilation parameters of the light and dark flash protocols, plotted against CVR. The light protocol a) dilation velocity (p = 0.668) and b) time to 75% recovery (p = 0.237) are shown on the left in blue. The dark protocol c) dilation velocity (p = 0.764) and d) dilation amplitude (p = 0.561) are shown on the right in red. Note that one subject is not included in the light flash plots as they did not have a complete dataset for their right eye in the light dilation parameters, due to blinking and other artefacts.*

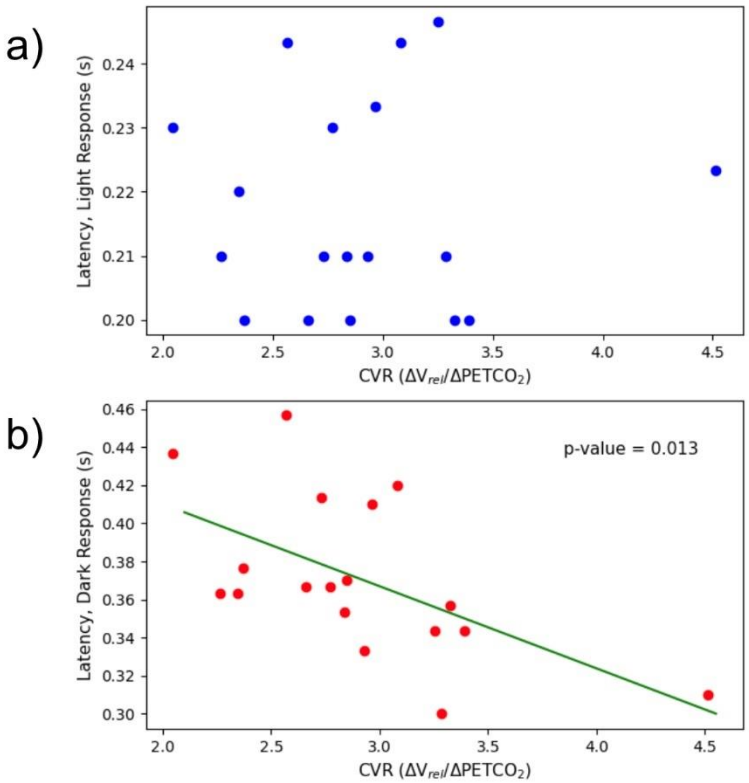

***Figure 4.6**: Latency plotted against CVR. This includes the latency in both a) the light flash protocol (p = 0.902) and b) the dark flash protocol (p = 0.0127) compared to the CVR.*

There were no statistically significant linear relationships between the constriction or dilation parameters of the PLR and CVR. There was, however, a statistically significant negative trend ($p = 0.0127$) with the latency in the dark flash protocol and CVR. There was no trend between the latency in the light flash protocol and CVR.

4.5. Discussion

To the authors' knowledge, this is the first study to provide an analysis of CVR and PLR measurements taken together.

CVR was calculated based on mean MCA blood velocity at baseline and during the inhalation of air with 5% CO_2 gas. The relative as opposed to absolute change in mean blood velocity between baseline and 5% CO_2 was used as the measure of interest for the CVR calculation, as this approach mitigates differences in probe location and angle that inherently occurs when collecting TCD data from different subjects. Participants were told to relax and breathe normally throughout the gas stimulus procedure and minimal differences were observed in the breathing rate and heart rate between the baseline and 5% CO_2 gas periods.

It should be noted that the cerebrovascular response is entangled with physiological mechanisms that affect cerebrovascular function including ventilatory sensitivity, chemoreflexes, and nitric oxide (NO). NO bioavailability has been shown to be a key contributor to cerebral shear-mediated dilation [301], however some studies have shown that NO synthase inhibition does not influence CVR based on a steady-state CO_2 stimulus [302], [303].

The CVR response to the inhalation of air with 5% CO_2 can also be impacted by the sensitivity of chemoreflexes including central and peripheral chemoreceptors, central nervous system, and ventilatory response to $PaCO_2$ [304], [305]. Notably, the sensitivity of central chemoreflexes in response to changes in $PaCO_2$ can differ between subjects and alter their ventilatory response [306]. An increase in the ventilatory response to CO_2 is especially pronounced when CBF is reduced such as in subjects with congestive heart failure and sleep apnoea [307] and changes in breathing could significantly alter CBF and $PaCO_2$ measures. As a result, despite relatively constant breathing rate and heart rate in our study, the CVR response is likely in-part also representative of chemoreceptor and ventilatory sensitivity [304].

As a result of the complex interplay between these mechanisms, vascular smooth muscle function is unlikely to be the only contributor to the CVR response. CVR may still be a good method for characterising vascular smooth muscle cell function *in-vivo* [248], however the involvement of numerous mechanisms is still poorly understood in humans due to the experimental limitations of isolating independent involvement.

For the PLR analysis, we investigated several parameters relating to both constriction and dilation of the pupil, as there are opposing systems working in both the constriction and dilation phases. Pupillary constriction and dilation are controlled by a variety of physiological mechanisms and neural pathways, including opposing muscles and different branches of the autonomic nervous system. In particular, the parasympathetic/sphincter system dominates the constriction phase with negligible contribution from the sympathetic/dilator system, while both systems contribute to

the beginning of the dilation phase [268]. This means that it is difficult to isolate the specific contributions of smooth muscle alone on various parameters of the PLR, as the smooth muscle dynamics relate strongly to contributions from the sympathetic and parasympathetic nervous systems. Despite the complexities associated with disentangling these relative contributions, assessing specific parameters of the PLR in relation to CVR can potentially provide a valuable insight into the relationship between the eye and the brain.

The average dilation velocity in the light flash protocol (1.22 ± 0.21 mm/s) was consistently smaller than the average dilation velocity in the dark flash protocol (1.61 ± 0.31 mm/s) and this difference was statistically significant ($p << 0.001$). This could be due to the nature of the protocols. In the light flash protocol, the stimulus first elicits a greater contribution from the sphincter/parasympathetic system to cause constriction, which is likely still active to a certain extent when the dilator/sympathetic system works to dilate the pupil post-stimulus. In the dark flash protocol, however, the stimulus first elicits a contribution from the dilator/sympathetic system, which would explain the larger magnitude of dilation velocity. Additionally, the pupil is only moderately constricted during the light flash protocol when it first begins to dilate, compared to the highly constricted pupil in the dark flash protocol, which would also support a smaller dilation velocity.

The latency of the dark flash protocol (mean = 0.37 ± 0.04 s) was consistently larger than that of the light flash protocol (mean = 0.22 ± 0.02 s). Conversely, the time to change response directions after the end of the second stimulus, was consistently larger in the light flash protocol than in the dark flash protocol – this demonstrates

that the latency in response to a loss of light was larger than that in response to the onset of light.

When comparing the PLR to CVR measurements, most constriction and dilation PLR parameters did not yield statistically significant results. The maximum constriction velocity and the time to 75% recovery showed negative trends associated with CVR, but these were not statistically significant.

Interestingly, there was a significant negative trend relating the latency in the dark flash protocol to CVR ($p = 0.0127$). In contrast, no statistically significant trend was observed with the latency in the light flash protocol and CVR. However, the range of values in latency for the light flash protocol, was significantly smaller than that of the dark flash protocol, which might partially explain the lack of trend. In the dark flash protocol, this statistically significant negative relationship between the pupillary latency and CVR, implies that with a higher CVR, the latency, or time to react to a stimulus change, is smaller. However, if accounting for multiple comparisons, the dark latency falls just outside of statically significant, therefore additional data and tests are necessary to confirm any significance of the results. Further research is warranted into pupillary parameters of the dark flash protocol, as this protocol has been less studied than the standard light flash protocol.

4.5.1. Limitations & Future Work

For CVR assessment, we used a 2-point CVR measure as this is the most common method for deriving CVR using TCD [249], [308], [309]. This strategy assumes a linear relationship between CVR and changes in $P_{ET}CO_2$. Although, it is known that CVR response is in fact sigmoidal in shape [261], [310], given our small dynamic range in

CVR and $P_{ET}CO_2$ measurements, we expect that our results fall within the linear range of the sigmoidal curve [254], [311]. Nevertheless, future research is warranted to further explore more descriptive models of the response of cerebral VSMCs to vasoactive stimuli.

It was also assumed that a steady state was achieved after 30 seconds of breathing the 5% CO_2 gas. While we know that $P_{ET}CO_2$ can continue to increase over even a 10 minute period [312], minimal change occurs after the 1st minute and a long period of breathing air with increased levels of CO_2 can be challenging for participants. Therefore, to maintain a clinically viable vasoactive stimulus, 3 minutes was agreed upon as a reasonable upper limit for most volunteers to comfortably breathe 5% CO_2.

Another possible limitation of the gas protocol was leakage of room air into the face mask during the gas stimulus which was an issue for some participants since the standardised mask did not create a tight seal with all face shapes. Minimal leakage of the 5% CO_2 gas mixture is visible in Figure 4.2 by the drops in the CO_2 trace during the inhales (troughs). Worse leakage was mitigated by using only one ventilation valve which was often one site of room air entry and refitting the mask to ensure no gaps were left around the participant's nose and mouth.

While a baseline blood pressure measurement was taken for all subjects using an arm cuff to rule out hypertension, continuous arterial blood pressure (ABP) measurements were not acquired in this study. Some studies have shown that changes in ABP, both spontaneous or induced by the inhalation of air with increased CO_2, can impact CBF velocity in response to vasoactive stimuli and therefore CVR in

some adults [313], [314], [315]. However, other studies have shown that even when using air with up to 7% inspired CO_2, the increase in ABP has minimal effects on MCAv and CVR [316]. Notably, Dumville *et al*. also reported that in healthy adults with no vascular disease and intact cerebral autoregulation, the CVR assessment as determined by the relative changes in velocity and $PETCO_2$ are independent of ABP provided that the pressure change is contained within the autoregulatory plateau [317]. This was echoed by Battisti-Charbonney *et al*., who showed that the MCAv response to CO_2 was unchanged by ABP considerations up to a threshold of approximately 50 mmHg, above which both MCAv and ABP appeared to increase linearly with CO_2 tension [318]. However, in patients with pathophysiology such as carotid artery disease, ABP has been shown to significantly alter CVR index calculations in response to inhalation of air with 5% CO_2 [317]. In our study, assessing only healthy adults below that threshold (maximum $PETCO_2$ of 46.5 mmHg) when undergoing the gas stimulus, the effects of ABP on our CVR calculations are assumed to be negligible. None-the-less, future studies may benefit from including continuous ABP monitoring (such as by using finger photoplethysmography or more accurately using an arterial catheter) during gas stimulus protocols, especially when investigating pathology.

Furthermore, regional differences in CVR are likely to exist throughout the brain [319], [320], [321], therefore CVR values based on the blood velocity measures in the MCA alone may only be representative of the brain regions supplied by the artery and may not illustrate cerebrovascular function in other regions of the brain.

Lasting cerebrovascular responses triggered during hypercapnic challenges can take additional time to return to baseline post-stimulus, and although the pupillometry was done at least 10 minutes after the gas stimulus for each participant, there is a small chance that there were still residual hypercapnic effects while the beginning of the pupillometry protocols were being performed. In the future, the PLR data could be collected prior to the gas stimulus. Alternatively, in a larger study cohort, the sequence of protocols could be swapped in half of the study cohort to clarify the PLR without the potential contamination of the after-effects of hypercapnia.

There are also some technical limitations that might have impacted the PLR data collected. Firstly, the frame rate of the NeurOptics pupillometer is low, with only 30 frames per second (i.e., 0.033 seconds per measurement). When comparing this to the entire range of average latency values in the light flash protocol, which is 0.043 seconds, this shows that the range of values is comparable to the sampling period of the device. The latency in the dark flash protocol avoids this problem due to the larger magnitude and range of values. With a smaller sampling period, there is potential that a trend could be identified in the light flash latency – this could not be investigated with the limitation of the current equipment. In future experiments, equipment with a higher frame rate should be used to thoroughly investigate any trends between the light flash latency and CVR.

An additional limitation was the assessment of the time to 75% recovery in the light flash protocol. The protocol only included 7 seconds of recovery time post-stimulus, as it was important to ensure that the entire protocol was short enough so that participants could withstand not blinking for the duration of each trial. In some

cases, however, 7 seconds was not enough time for subjects to recover to 75% of their baseline, initial pupil diameter. When the pupil did not recover to 75% of its initial diameter, no value was reported for this parameter, reducing the number of trials to be included in the average. Additionally, if the subject blinked, the time to 75% recovery and dilation velocity parameters were also not calculated – this also reduced the number of trials included in the analysis for some subjects. In the future, using equipment that can remove blinking artefacts in the data, which would enable a longer recovery time to be included in the analysis, would enable a more confident assessment of dilation parameters in the light flash protocol – especially with the time to 75% recovery, where we would expect to see some higher values recorded.

Although CVR and certain PLR metrics are known to be dependent on age [322], [323], [324] and sex [325], [326], [327], we did not observe significant differences between ages and sexes. This is likely explained by our small sample size of groups, and as a result statistics could not be confidently performed on the influences of sex and age.

In the future, we plan to increase the dynamic range in vasoactive stimuli, vary the light stimuli for the eye, and improve the imaging resolution for both the blood flow measures and pupil measures. Notably, independently repeating the experiment of the dark flash protocol is necessary to confirm any significance in latency correlating with CVR. This analysis will take place in a larger participant group with a wide range of ages, lifestyle factors, and demographics for a more robust statistical analysis of the interplay between cerebral blood flow and pupil dynamics.

4.6. Conclusion

In this work, we compared the pupillary light response in light and dark flash protocols, to cerebrovascular reactivity assessed using transcranial Doppler ultrasound, to investigate the relationship between dynamics in the eye and brain. We found a significant negative relationship between the latency of the PLR in the dark flash protocol and CVR. No statistically significant relationships were found between CVR and other PLR metrics. This is the first study that has investigated the relationship between cerebral blood flow and pupil dynamics. Future work will incorporate other protocols and equipment, in both pupillometry and in CVR assessment, that might retrieve additional information of interest and further control for confounding factors. Furthermore, a broader range of subjects across age, health, and lifestyle factors will be considered to investigate the validity of these relationships when subject to a larger dynamic range of subjects.

Chapter 5: Investigating the pupillary light response in the postpartum period

This chapter aims to understand the relationship between the PLR in two groups of women, by evaluating the impact of an important sex-specific factor that may mediate this relationship: pregnancy. Although previous work in Chapter 4 did not identify significant differences across males and females in their PLR, there may be intra-sex differences based on other factors, such as pregnancy. The impact of pregnancy on the PLR has not been investigated, nor has its impact on many other metrics related to neurodegeneration risk been evaluated before this study.

Importantly, the data collected and presented in this chapter informs the analysis conducted in the final chapter, and highlights the importance of considering demographic factors, including sex-specific factors such as pregnancy history, on the pupillary light response as well as on other covariates and contributing factors.

Although sometimes used interchangeably, the concepts of sex and gender have specific definitions and unique implications that should be considered independently in health research [328], [329] – sex refers to biological attributes that encompass anatomy, physiology, genes, and hormones, while gender refers to social and cultural constructed roles and identities of women, men, girls, boys, and gender diverse people [330], rooted in biology but shaped through environment and experience [331]. Both sex and gender, although separate concepts, are linked [328], and are both determinants of health [332].

In this chapter, we will focus on the biological sex of an individual, defined by their reproductive anatomy and physiology at birth. The word “women” is used in this chapter’s context to describe people who were born with a female reproductive system or whose sex assigned at birth was female.

This chapter relates to Research Questions 1 and 3, is currently a manuscript in preparation for submission, and includes work from the following studies:

Sparks, S; Bulte, DP; Hayes, G; Pinto, J (2025). “Investigating the pupillary light response in the postpartum period”. Association for Research in Vision and Ophthalmology Annual Meeting 2025. Accepted abstract.

Pinto, J; Suri, S; **Sparks, S;** Hayes, G; Bulte, DP (2025). “Cerebrovascular reactivity dynamics in the postpartum period: a pilot study”. International Society for Magnetic Resonance in Medicine Annual Meeting 2025. Accepted abstract.

Pinto, J; Suri, S; **Sparks, S;** Hayes, G; Bulte, DP (2025). “Cerebrovascular reactivity in the postpartum period: exploring regional differences using fMRI”. Organisation for Human Brain Mapping Annual Meeting 2025. Accepted abstract.

Author contributions: S. Sparks contributed to the pupillometry study concept and design, pupillometry methods development, data acquisition, analysis and interpretation, statistical analyses, manuscript idea conception, and writing and editing of the manuscript. G. Hayes contributed to data acquisition. D. Bulte contributed to revision of the manuscript. J. Pinto contributed to ethics approvals,

original study concept and design, data acquisition, revision of the manuscript, and study supervision.

5.1. Introduction

5.1.1. Background and Motivation

Sex differences exist in the risks and manifestation of many diseases [328], [329], [330], [331], [332]. An example of sex and gender interactions on health includes Alzheimer's disease, where women are approximately twice as likely as men to be diagnosed with the disease [333], [334], [335]. This cannot be explained by the increased life expectancy of women alone [212] and could be due to biological factors, social factors, or an intersection of both [336], [337], [338]. Despite this evidence of sex-specific differences in risk for diseases such as Alzheimer's disease, women's health continues to be understudied, particularly in neuroscience studies [339]. Not only have females been historically excluded from preclinical research and biomedical studies, even more recent studies also often overlook sex-specific variables, which Taylor *et. al.* claim is "a bias that seeps into our study design and analyses and impedes our basic understanding of the brain" [340]. With research demonstrating differences in the male and female brain [341], as well as with sex and gender differences in the development and prognosis of its diseases, it is essential to incorporate a consideration of sex-specific factors for a more complete understanding of the brain and its processes.

One sex-specific event that may impact metrics of brain health is pregnancy, however this has not been studied at-large. Research has shown that some aspects of pregnancy, including the number of pregnancies, may be related to cognitive

status and Alzheimer's disease risk [342], [343], [344], and more research is needed to investigate pregnancy-related metrics on other aspects of women's overall and cerebral health.

There are known ocular changes that occur in pregnancy, including refractive status, cornea sensitivity, visual acuity, and intraocular pressure [345], [346], [347]. However, many of these changes are transient [348], [349] and return to their prepregnant state several weeks postpartum [350], potentially due to hormonal changes that peak during pregnancy but return to pre-pregnancy levels during the postpartum period [345]. Interestingly, the pupillary light response (PLR), which is controlled by the autonomic nervous system and has been reported to change in cases of neurodegeneration [83], [92], [94] and in those at-risk for neurodegeneration [245], has not been investigated in either pregnant or postpartum women. Additionally, changes to the autonomic nervous system, particularly increased sympathetic nervous system activity, have been reported in pregnant subjects [351], [352], [353], however longer-term impacts in the postpartum period remain understudied. With these established ocular and autonomic response changes that occur in pregnancy period, and the relation between the pupillary light response, autonomic function, and neurodegeneration, there is an opportunity to explore these factors outside of the pregnancy period in postpartum women. In particular, assessing the pupillary light response to identify potential changes to the nervous system, as well as potential links to Alzheimer's disease risk, in postpartum women.

This study aims to explore the relationship between metrics of the PLR in postpartum women and women who have never been pregnant, to investigate changes that occur to the PLR in relation to pregnancy.

5.2. Methods

5.2.1. Subjects

We acquired data from 23 healthy females, with no history of neurological disorders (age range 25-43 years, with a mean age of 32.5 + 5.5 years at the time of acquisition). Inclusion criteria consisted of being assigned female at birth, having no diagnosed cognitive impairment, no severe respiratory conditions, and no visually triggered migraines or epilepsy. Participants with corrective prescription glasses did take part in the study, but none who had known vision loss and none who had undergone eye or brain surgery.

Participants were screened for either the postpartum group (given birth within the past two years), or the control group (no history of pregnancy), with the control group recruited to be age-matched to the postpartum group. Within the postpartum group, subjects were further classified as having normotensive (n = 9) or hypertensive pregnancies (n = 4); this information was not used for statistical analyses due to the small number of hypertensive pregnancies. All participants provided informed written consent before each session, and the study was approved by the Medical Sciences Interdivisional Research Ethics Committee (MS IDREC) of the University of Oxford's Central University Research Ethics Committee (CUREC).

5.2.2. Data Acquisition

5.2.2.1. Pupillometry

Pupillary light response data were acquired with the NeurOptics PLR-3000 hand-held pupillometer (NeurOptics, Irvine, CA). This automated, monocular pupillometer uses an infrared camera to capture and measure the pupil size, allowing it to be used in both light and dark settings, and is widely used in clinical practice and research settings.

There were two protocols used to assess the pupillary light response, which were done with a 2-minute period of dark adaptation in a dark, quiet testing room. Both protocols were previously used by the researchers in similar studies assessing the PLR and metrics of brain function, and are fully described in previous work [16].

For each subject, six measurements were performed on each eye, alternating eyes between each trial, starting with the right eye. The first three measurements on each eye, the light flash protocol, were with the positive step-input stimulus, which had a 1 s baseline measurement, a 1 s flash of 50 μW white light, and 7 s of post-stimulus measurement, with a 1-minute interstimulus interval. The last three measurements on each eye, the dark flash protocol, were with the negative step-input stimulus, which had a 1 s baseline measurement with the 50 μW light on, a 1 s dark flash with the light off, and a 7 s measurement with the light back on, also with a 1-minute interstimulus interval.

During the measurements, subjects were instructed to keep their eyes wide open and to avoid blinking, and to hold a constant gaze position. The pupillometer was

held at a right angle to the subject's line of sight. All measurements were taken between 09:00 and 17:00 to avoid interference from circadian rhythms.

5.2.2.2. Additional Information

To account for potential covariates other than postpartum status, age, months postpartum (for the postpartum group), and several physiological measurements were used to compare their effects on the PLR in both the postpartum and control groups. The physiological measurements, all of which were taken before any pupillometry experiments, were done during data acquisition and included body mass index (BMI), blood pressure, and resting heart rate. Three blood pressure measurements were taken using an automatic blood pressure monitor whilst sitting, which also recorded heart rate. Systolic and diastolic blood pressure measurements were averaged across trials, and the average of the heart rate measurements was used as the resting heart rate.

Table 5.1 outlines the subject demographics from this study, with means and standard deviations presented.

Table 5.1: *Subject Demographics for the Control and Postpartum Groups*

Parameter	Control (n = 10)	Postpartum (n = 13)	p value
Age (years)	31.0 ± 6.7	33.6 ± 4.3	0.27
Post-partum period (months)	NA	14.2 ± 6.2	NA
BMI (kg/m^2)	26.1 ± 4.9	25.6 ± 5.5	0.83
Systolic Blood Pressure (mmHg)	99.5 ± 9.1	105.6 ± 12.4	0.21
Diastolic Blood Pressure (mmHg)	71.4 ± 8.6	74.8 ± 12.5	0.47
Resting Heart Rate (bpm)	73.8 ± 11.0	73 ± 9.9	0.86

5.2.3. Data Analysis

5.2.3.1. Pupillometry Analysis

The time-course data from the pupillometry experiments (sampled at 30 Hz) were extracted directly from the NeurOptics PLR-3000 pupillometer in a CSV file format and processed using custom scripts in Python.

As defined in previous work [16], the NeurOptics pupillometer automatically calculates several metrics of the pupillary light response in both the light and dark flash protocol for each 9 s measurement. After removing trials that were not usable due to blinking artefacts, values were averaged across all trials for each participant.

Due to the nature of the PLR protocols, constriction parameters, dominated by the sphincter muscles and parasympathetic nervous system, were only assessed in the light flash protocol, as the dark flash protocol's constriction amplitude was significantly smaller than that of the light flash protocol where the pupil starts at a larger, dark-adapted diameter, as previously reported [16]. Dilation parameters, however, were assessed in both the light and dark flash protocols, and have contributions from the dilator muscles and sympathetic nervous system as well as the sphincter muscles and parasympathetic nervous system [68].

For the light flash protocol, the key parameters that were assessed were (a) the average constriction velocity, (b) the maximum constriction velocity, (c) the constriction amplitude, (d) the dilation velocity, (e) the time to 75% recovery, and (f) the latency of the response. For the dark flash protocol, the key parameters assessed were (g) the dilation velocity, (h) the dilation amplitude, and (i) the latency of the response. These parameters are as previously defined in Figure 4.1.

5.2.3.2. Comparison Analysis

To identify any statistically significant relationships between parameters of the PLR across the postpartum and control groups, we performed tests for normality for each PLR parameter using the Shapiro-Wilk test in each group, where parameters were said to be normally distributed if $p > 0.05$. For each PLR parameter that was normally distributed across both groups, we performed one-way ANOVA tests (significance level $p < 0.05$, uncorrected). For all other PLR parameters, we performed Kruskal-Wallis tests (significance level $p < 0.05$, uncorrected).

To account for covariates, we compared each metric of the PLR against the group and the following physiological metrics chosen as covariates, using ANCOVA Type 2 analysis: age, BMI, months postpartum (for the postpartum group only), systolic blood pressure, diastolic blood pressure, and resting heart rate. This was done without further interactions between factors, and is described in equation 5.1:

$$\{\boldsymbol{each\ PLR\ metric}\} \sim \boldsymbol{Group} + \{\boldsymbol{covariates}\}, \boldsymbol{where}$$
$$\boldsymbol{covariates} = \boldsymbol{age} + \boldsymbol{BMI} + \boldsymbol{postpartum\ months} + \boldsymbol{BP_{sys}} + \boldsymbol{BP_{dias}} + \boldsymbol{RHR} \quad \textit{(eq. 5.1)}$$

Further, a correlation matrix was generated across all PLR and defined physiological metrics using Pearson correlation analysis, combining all subject data.

5.3. Results

All 23 subjects completed the full pupillometry protocols and completed basic physiological measurements.

5.3.1. Pupillometry Results

As outlined in the Methods, data were collected from both the right and left eyes of each participant. Two participants did not have usable data for their right eye in the light flash protocol, due to blinking at the light flash across trials. Due to data completeness, the left eye of all subjects was used for further analysis. This was also to ensure that averaging across both eyes did not introduce any artefacts.

Overall, the pupillary light response characteristics were as expected and comparable to previous studies [16], [354]. As shown in a previous study with the same NeurOptics protocol [16], the interstimulus interval selected allowed for sufficient time for the pupil to recover to its baseline size before subsequent trials.

Of the PLR parameters in the light flash protocol, all variables were normally distributed and used one-way ANOVA for statistical comparisons, except for the end diameter and latency which were not normally distributed and thus used the Kruskal-Wallis H-test. For the dark flash protocol, all parameters were normally distributed and used one-way ANOVA for statistical comparisons, except for the latency and dilation velocity which were not normally distributed and thus used the Kruskal-Wallis H-test.

Tables 5.2 and 5.3 show the average values for the PLR metrics across both groups of women, in the light and dark flash protocols respectively.

***Table 5.2**: Pupillary light response metrics for the light flash protocol, left eye, not controlling for covariates*

	Control Mean ± Std Dev	Postpartum Mean ± Std Dev	p value
Initial Diameter (mm)	6.43 ± 0.79	6.29 ± 0.51	0.61
End Diameter (mm)*	3.55 ± 0.61	3.56 ± 0.46	0.98
Latency (s)*	0.21 ± 0.01	0.21 ± 0.03	0.66
Constriction Velocity (mm/s)	-3.11 ± 0.40	-2.95 ± 0.52	0.42
Max Constriction Velocity (mm/s)	-5.53 ± 0.72	-5.18 ± 0.82	0.31
Dilation Velocity (mm/s)	1.15 ± 0.21	1.16 ± 0.30	0.93
Time to 75% Recovery (s)	4.70 ± 0.68	4.81 ± 0.64	0.70
Amplitude of Constriction (mm)	2.88 ± 0.39	2.73 ± 0.37	0.35

**: not normally distributed*

***Table 5.3**: Pupillary light response metrics for the dark flash protocol, left eye, not controlling for covariates*

	Control Mean ± Std Dev	Postpartum Mean ± Std Dev	p value
Initial Diameter (mm)	2.81 ± 0.40	2.88 ± 0.26	0.62
End Diameter (mm)	3.88 ± 0.55	3.87 ± 0.33	0.94
Latency (s)*	0.36 ± 0.04	0.37 ± 0.04	0.60
Dilation Velocity (mm/s)*	1.65 ± 0.56	1.36 ± 0.29	0.26
Amplitude of Dilation (mm)	1.07 ± 0.22	0.99 ± 0.17	0.32

**: not normally distributed*

There were no statistically significant differences between the postpartum and control groups in any of the PLR parameters across both the light and dark flash protocols (not controlling for covariates). However, a weak trend towards a decreased dilation velocity in the dark flash protocol for the postpartum group was observed ($p = 0.26$), but not in the light flash protocol ($p = 0.93$).

5.3.2. Comparison of Pupillometry and Covariates

Each parameter of the PLR was compared against age, postpartum months, and previously defined physiological measurements, which were used as covariates. A correlation matrix combining all subject data for the light and dark flash protocols, using the Pearson correlation relationship, is shown in Tables 5.4 and 5.5, with statistically significant correlations bolded.

***Table 5.4**: Pearson correlation and corresponding p values for pupillary light response metrics for the light flash protocol (left eye) and covariates*

	Age	BMI	Postpartum Months	Systolic Blood Pressure	Diastolic Blood Pressure	Resting Heart Rate
Initial Diameter (mm)	-0.38 (p = 0.206)	0.23 (p = 0.453)	0.16 (p = 0.605)	0.23 (p = 0.458)	0.28 (p = 0.348)	0.09 (p = 0.760)
End Diameter (mm)*	**-0.65 (p = 0.017)**	0.20 (p = 0.522)	-0.09 (p = 0.768)	0.06 (p = 0.835)	0.19 (p = 0.545)	-0.11 (p = 0.726)
Latency (s)*	-0.09 (p = 0.765)	-0.12 (p = 0.690)	-0.05 (p = 0.881)	**-0.67 (p = 0.011)**	**-0.65 (p = 0.016)**	-0.47 (p = 0.101)
Constriction Velocity (mm/s)	-0.35 (p = 0.244)	0.01 (p = 0.961)	-0.35 (p = 0.243)	-0.34 (p = 0.252)	-0.27 (p = 0.379)	-0.34 (p = 0.254)
Max Constriction Velocity (mm/s)	-0.22 (p = 0.467)	-0.18 (p = 0.557)	-0.08 (p = 0.798)	-0.40 (p = 0.172)	-0.45 (p = 0.121)	**-0.56 (p = 0.047)**
Dilation Velocity (mm/s)	0.17 (p = 0.571)	0.22 (p = 0.480)	-0.20 (p = 0.516)	-0.05 (p = 0.870)	0.17 (p = 0.572)	0.18 (p = 0.550)
Time to 75% Recovery (s)	-0.16 (p = 0.606)	0.39 (p = 0.192)	0.22 (p = 0.477)	0.23 (p = 0.448)	0.48 (p = 0.096)	**0.65 (p = 0.015)**
Amplitude of Constriction (mm)	0.28 (p = 0.362)	0.07 (p = 0.808)	0.33 (p = 0.268)	0.23 (p = 0.442)	0.16 (p = 0.592)	0.26 (p = 0.385)

***Table 5.5**: Pearson correlation and corresponding p values for pupillary light response metrics for the dark flash protocol (left eye) and covariates*

	Age	BMI	Postpartum Months	Systolic Blood Pressure	Diastolic Blood Pressure	Resting Heart Rate
Initial Diameter (mm)	**-0.60 (p = 0.030)**	0.26 (p = 0.399)	0.02 (p = 0.951)	0.24 (p = 0.437)	0.39 (p = 0.186)	0.08 (p = 0.787)
End Diameter (mm)	-0.44 (p = 0.133)	0.39 (p = 0.191)	-0.00 (p = 1.000)	0.28 (p = 0.356)	0.43 (p = 0.143)	0.21 (p = 0.499)
Latency (s)*	-0.06 (p = 0.844)	0.26 (p = 0.389)	-0.34 (p = 0.255)	-0.41 (p = 0.160)	-0.39 (p = 0.193)	-0.52 (p = 0.067)
Dilation Velocity (mm/s)*	-0.01 (p = 0.980)	0.48 (p = 0.098)	-0.05 (p = 0.884)	0.01 (p = 0.975)	0.13 (p = 0.673)	0.29 (p = 0.335)
Amplitude of Dilation (mm)	0.05 (p = 0.882)	0.37 (p = 0.213)	-0.03 (p = 0.926)	0.19 (p = 0.537)	0.25 (p = 0.412)	0.28 (p = 0.362)

From the ANCOVA analysis, after controlling for covariates as defined in Equation 5.1, there were additional relationships identified. From the light flash protocol, the group (either postpartum or control) had statistically significant impacts on initial

diameter, end diameter, and latency ($p = 0.02$, $p = 0.005$, and $p = 0.01$, respectively). Notably, age also significantly impacted the end diameter ($p = 0.04$). From the dark flash protocol, the group had statistically significant impacts on the same PLR parameters (initial diameter, $p = 0.002$; end diameter, $p = 0.02$; latency, $p = 0.02$), with age significantly impacting the initial diameter in this protocol ($p = 0.03$). Full ANCOVA tables are shown in Appendix B.

5.4. Discussion

To the authors' knowledge, this is the first study to provide an analysis of the pupillary light response in postpartum women compared to women who have never been pregnant.

The light and dark flash protocols used for the pupillary light response evaluation allowed for the analysis of constriction, dilation, and latency parameters. Pupillary constriction and dilation have inputs from branches of the autonomic nervous system and evaluating differences across postpartum and control groups in these parameters can provide an initial consideration of potential differences in these neural pathways. Although generally, the parasympathetic system mediates pupillary constriction and the sympathetic system mediates pupillary dilation, there are interactions between both systems in the pupillary light response [355], [356]; a consideration of both the light and dark flash reflex as demonstrated with the two protocols used allows for greater consideration of these interactions.

From the initial analysis without consideration of covariates, there were no statistically significant differences identified across the postpartum and control groups in any of their PLR parameters.

Interestingly, there was a weak trend towards a decreased dilation velocity in the dark flash protocol for the postpartum group ($p = 0.26$), but no notable differences in the dilation velocity across groups in the light flash protocol ($p = 0.93$). As the protocols have different autonomic innervations, this emphasizes the importance of investigating the PLR with both the light and dark flash protocol to identify subtle differences across groups, particularly to identify potential nervous system differences. This result highlights possible differences in autonomic responses postpartum, particularly in the sympathetic branch which contributes to pupillary dilation. Changes to the autonomic nervous system have been shown in pregnancy, including sympathetic control and vascular responsiveness [351], [352]. However, the changes that occur in pregnancy generally include an increase in sympathetic activity, rather than a decrease as the results here suggest [353]. This may indicate differences in autonomic system compensation between pregnant and postpartum states. Given the impacts of pregnancy on the autonomic nervous system and the results of this study, changes to the PLR in the postpartum period should be investigated in a larger group of subjects to test for significance.

Although there have not been studies identified by the authors that compare the dynamic factors associated with the PLR in pregnancy or postpartum periods, one study was identified which noted no statistically significant differences across postpartum (within 2 months of childbirth) and control groups in their light-adapted pupil sizes [345]. This study, which did not account for covariates, agrees with the findings of our study before accounting for covariates.

Latency in the light flash protocol was negatively correlated with both systolic blood pressure ($r = -0.67$, $p = 0.011$) and diastolic blood pressure ($r = -0.65$, $p = 0.016$). Time to 75% recovery was correlated with the measured resting heart rate ($r = 0.65$, $p = 0.015$). Maximum constriction velocity was negatively correlated with resting heart rate ($r = -0.56$, $p = 0.047$). Finally, age was negatively correlated with the end diameter in the light flash protocol ($r = -0.65$, $p = 0.017$). In the dark flash protocol, the only additional relationship identified was between initial diameter and age, which displayed a negative correlation (-0.60, $p = 0.030$).

From previous literature, pupil size is known to decrease with age, which is most pronounced at lower illuminance levels [67], [357], [358]. Interestingly, this relationship was only statistically significant in the end pupil diameter for the light flash protocol and the initial pupil diameter for the dark flash protocol, both of which correspond to the highest illuminance cases. Although there were negative trends associated with the lower illuminance pupil diameters, these did not reach statistical significance. This discrepancy may be due to the relatively small age range of our cohort.

After controlling for covariates with the ANCOVA analysis, additional relationships were identified. Across both the light and dark flash protocols, being in either the postpartum or control group statistically significantly impacted initial pupil diameter, end pupil diameter, and latency of the response, after controlling for covariates. Of the covariates, age was most important and significantly impacted both the end diameter of the light flash protocol and the initial diameter of the dark flash protocol, a result consistent with the Pearson correlation analysis of these variables. Further,

the difference between dilation velocity in the dark flash protocol was no longer statistically significant after controlling for these covariates, demonstrating that the proposed sympathetic changes seen in the postpartum women may, importantly, be mediated by other physiological factors.

This covariate analysis shows that the categorical factor of postpartum or control does have impacts on both static (pupil size) and dynamic (latency) parameters of the PLR when accounting for age, basic physiological factors, and postpartum months. Notably, the latency of the dark flash protocol has previously been shown to be negatively correlated with cerebrovascular reactivity, an important metric of brain vascular health [16]. This suggests that there may be changes in brain vascular health and hemodynamics in postpartum women, in addition to the known changes in hemodynamics that occur during pregnancy [352]. Further research investigating the impact of pregnancy and the postpartum period, on metrics of hemodynamics and brain vascular health such as CVR, is warranted.

Importantly, the number of months postpartum did not show any statistically significant impacts on, or relationships with, any parameters of the PLR. None of the participants were less than four months postpartum, and so this suggests that demonstrated changes in parameters of the PLR in postpartum women are likely attributed to lasting changes associated with a history of pregnancy, rather than associations with the transient period between pregnancy and early postpartum weeks and months.

5.4.1. Limitations and Future Work

As previously outlined, there are limitations with the NeurOptics PLR-3000 pupillometer and methods – notably, that the frame rate of the pupillometer is relatively low (30 frames per second), and that the 7 s recovery time is not always sufficient time for subjects to reach 75% recovery [16].

The measurements for blood pressure and resting heart rate had limitations due to the equipment and protocols used. Three measurements were taken from the automated blood pressure monitor to account for normal variability in blood pressure [359] and heart rate [360], however both blood pressure and heart rate can be elevated in individuals who feel nervous or agitated . Data collection can be stressful, especially for those who are not used to scientific studies, and this could have artificially elevated the blood pressure and heart rate values. To get a more reliable measure of these, resting heart rate should be acquired as the participant is truly resting if time permits, and blood pressure measurements should be taken both at the beginning and end of experimentation to determine whether there are significant changes observed.

An additional limitation is in the background of the postpartum group. For this group, the number of months postpartum was not consistent, some of the participants had more than one pregnancy, and there were also some participants that had complications (such as hypertensive disorders of pregnancy). Hypertensive disorders of pregnancy contribute to an increase in sympathetic nerve activity compared to normotensive pregnancies [351], and have been shown to cause structural brain changes [361] and greater brain atrophy compared to normotensive

pregnancies [362]. Given these factors, and that hypertensive pregnancies present additional risk for hypertension, cardiovascular diseases, and other comorbidities [363], [364], [365], the distinction between hypertensive and normotensive pregnancies is important in considering potential health outcomes. Although postpartum months were included in the covariate analysis to account for this potential impact on the results, the number of pregnancies and disorders of pregnancy were not included in the covariate analysis, due to the limited sample size. Future work should aim to standardize the number of pregnancies, and consider normotensive and hypertensive pregnancies separately, to further account for these covariates in the PLR-postpartum relationships.

Finally, BMI was used in this study as a means of categorizing the anthropometric height and weight characteristics of participants. Although BMI is a useful indicator connected to several health issues [366], [367], there have been criticisms on the accuracy of relying on BMI for health assessment. BMI is deficient in measuring fat and how it is distributed on the body, which plays a significant role in cardiometabolic disorders [367]. Metrics involving waist circumference indices have been shown to outperform BMI in assessing cardiometabolic risk [367], [368], [369], and future work could employ some of these indices, such as the ratio of waist to hip circumference, to have a better assessment of how fat distribution and potential cardiometabolic risk affect the PLR in postpartum women.

5.5. Conclusion

In this work, we compared the pupillary light response in light and dark flash protocols across two groups of women: those within two years of the postpartum

period, and those who had never been pregnant. To the authors' knowledge, this is the first work investigating the relationship between the extended postpartum period and the PLR. Without accounting for covariates, dilation velocity in the dark flash protocol was smaller (although not significantly so) in the postpartum group, indicating a potential impact of pregnancy on sympathetic innervation in the postpartum period. After controlling for covariates, light and dark-adapted pupil size, as well as latency of the pupillary light response in both protocols, were also significantly impacted by postpartum status, indicating that both static and dynamic components of the pupillary light response may be mediated by age and physiological metrics such as blood pressure, BMI, and resting heart rate.

This work highlights the need for more research on pupillary response and nervous system changes with pregnancy, in a larger cohort and during other pregnancy stages. Future work should also compare these metrics directly to cerebrovascular reactivity and other brain vascular health metrics, to extrapolate these findings to investigate the implications on neurodegeneration risk post-pregnancy.

Chapter 6: The sleep-mediated relationship between the pupillary light response and cerebrovascular reactivity

This chapter aims to bring together the methods and results from all previous chapters, to expand the investigation of the pupillary light response and cerebrovascular reactivity in a larger, more diverse group, using methods to assess both the PLR and the post-illumination pupil response. Chapter 2 presented some of the important lifestyle and demographic risk factors associated with Alzheimer's disease and how, if at all, they relate to the pupillary light response, and some of these factors have been included in the analysis for this chapter. Chapter 3 presented preliminary results from pupillometry methods testing, some of which will be used in this chapter. Chapters 4 and 5 used a light and dark flash protocol for assessing the PLR, which will be used here for continuity. Chapter 5 highlighted the importance of demographic and physiological factors when assessing metrics such as the PLR, which will be considered here. Finally, Chapter 4 presented an initial investigation of the relationship between the PLR and CVR in a small group, and this chapter aims to explore this relationship in a larger group with an investigation into some covariates which may contribute to this relationship.

This chapter will ultimately explore the PLR, PIPR, and CVR relationship, with a consideration for lifestyle and other factors that have been investigated in relation to neurodegeneration risk. The aim is to use the findings from this chapter to enable

future CVR-based Alzheimer's disease risk classification, using the PLR/PIPR, demographic and genetic factors, and lifestyle factors.

The focus of this chapter is to present the pupillometry results and analysis, as well as the consideration of sleep as a covariate, and relate these to CVR. For the purposes of this thesis, as the CVR calculations were led by another researcher, the full protocol for acquiring the linear CVR values from the TCD analysis has not been included here. However, this information is available upon request.

This chapter relates to Research Questions 2 and 3, and is currently the basis for a manuscript in preparation.

Author contributions: S. Sparks contributed to the pupillometry study concept and design, pupillometry methods development, data acquisition, analysis and interpretation, statistical analyses, manuscript idea conception, and writing and editing of the manuscript. G. Hayes contributed to the CVR study concept and design, acquisition, CVR processing and analysis. J. Pinto contributed to ethics approvals and data acquisition. D. Bulte developed the original study concept and design, revised the manuscript, and supervised this work.

6.1. Introduction

6.1.1. Background and Motivation

Both cerebrovascular reactivity (CVR), an important metric of brain vascular health, and the pupillary light response (PLR), a metric related to autonomic nervous system and smooth muscle function, have been shown to be impaired in overlapping pathologies including neurodegeneration [16], [51], [52], [370]. As shown in a

previous pilot study, there is a relationship between these metrics of brain health [16], which warranted further exploration in a larger, more diverse subject group to identify the impact of covariates and other factors on this relationship.

Chromatic pupillometry, an evaluation of the PLR at different wavelengths and intensities of light, can aid with differentiating specific photoreceptor contributions to the PLR – notably, melanopsin-dependent responses versus rod/cone-mediated responses [229], [231], [371], [372], [373]. Additionally, the dark adaptation level can change the sensitivity of different photoreceptors [374]. Chromatic pupillometry has been investigated in neurodegeneration studies [246], [375], [376], although not all studies have shown differences in pupillary responses to chromatic light stimuli between Alzheimer's disease patients and cognitively healthy controls [90]. The post-illumination pupillary response (PIPR) has also been shown to be related to cognition and neurodegeneration [377], [378]. Although the PIPR assessed with chromatic pupillometry has been compared with CVR in a small subject group previously [379], the trends approaching significance require further investigation in a larger, more diverse cohort – particularly in those with a range of demographic and lifestyle factors, to similarly identify the impact of covariates on the relationship observed between the PIPR and CVR.

One important factor in overall health that could act as a covariate to the PLR/PIPR and CVR relationship is sleep – both the duration and quality can have significant impacts on cognition and other aspects of life. Sleep disturbances and shortened sleep in the long term has been associated with an increased risk of dementia [380], [381]. Interestingly, sleep has additional relationships with both the pupillary

responses to light and CVR more directly. Attenuated PIPR has been linked with a decrease in the amplitude of rest-activity behaviours, such as sleep, in healthy adults [382], with similar decreases in rest-activity behaviours observed in individuals with Alzheimer's disease [382].

Due to previous work identifying potential relationships between the PLR, PIPR, and CVR, as well as the impact of sleep on cognition, pupillary light responses, and CVR, this work aims to bridge the gap and consider these factors simultaneously in a larger, more diverse cohort. Therefore, this pilot study aims to explore the sleep-mediated relationship between the PLR, PIPR, and CVR in adults, to investigate these relationships in a more diverse cohort.

6.2. Methods

All participants provided informed written consent before each session, and the study was approved by the Medical Sciences Interdivisional Research Ethics Committee (MS IDREC) of the University of Oxford's Central University Research Ethics Committee (CUREC).

6.2.1. Subjects

Data was acquired from 33 adult subjects with no record of neurological disorders (19F, age range 18 – 70 years, with a mean of 32.7 ± 10.1 years at the time of acquisition). Inclusion criteria consisted of having no diagnosed cognitive impairment, psychiatric conditions, or history of asthma, along with passing the Wellcome Centre for Integrative Neuroimaging 3T Safe Scanner list. Participants with corrective prescription glasses did take part in the study, but none who had known vision loss and none who had undergone eye or brain surgery.

6.2.2. Pupillometry Data Acquisition

There were two separate pupillometry setups for this study, to evaluate both the PLR and the PIPR.

6.2.2.1. PIPR with PyPlr

The first protocol assessed the PIPR in response to blue light and red light, using PyPlr, an open-source system of hardware and software developed in-house [18]. The system uses infrared cameras attached to the Pupil Core device (Pupil Labs) to assess the pupillary light response in both eyes simultaneously, with the light stimulus sent from the LED Light Engine (Light Engine Technologies). This setup is further described in Chapter 3 and the protocol is adapted from previous work [379].

Subjects first underwent a 2-minute dark adaptation in a dark, quiet testing room, before the light stimuli were administered. The PIPR protocol consisted of a 1 s blue flash (470 nm peak) followed by a 90 s interstimulus interval (ISI), then a 1 s red flash (659 nm peak, matched for unweighted irradiance) followed by a 90 s ISI, to assess the PIPR at maximal and minimal melanopsin sensitivity, respectively. Three trials were administered for this protocol, and responses were processed using custom Python code to remove high-frequency noise and blinking artefacts [230]. The spectral information is available in Figure A.2 of Appendix A.

6.2.2.2. PLR with NeurOptics

The second set of protocols involved the NeurOptics PLR-3000 hand-held pupillometer (NeurOptics, Irvine, CA), which has been described in previous chapters. A light flash protocol (positive stimulus) and dark flash protocol (negative stimulus), each with a 1 s stimulus and 60 s ISI, were done using the PLR-3000. Both

of these protocols were previously used by the researchers in similar studies assessing the PLR and metrics of brain function, and are fully described in previous work in Section 4.3.2.2 [16]. All measurements were taken between 09:00 and 16:00 to avoid interference from circadian rhythms.

6.2.3. Transcranial Doppler Ultrasound Data Acquisition

Cerebrovascular reactivity data was acquired using a transcranial Doppler ultrasound setup with a ramp gas challenge protocol. The protocol consisted of three "ramps" designed to linearly increase the end-tidal carbon dioxide ($P_{ET}CO_2$) with cerebral blood flow measured using TCD, where the subject breathed increasing amounts of carbon dioxide mixed with air, ranging from normal air to 10% CO_2. The full TCD protocol is described in previous work [383].

The linear CVR was used for further analysis, which was calculated as the slope of a straight line to the $P_{ET}CO_2$ (mmHg) vs. TCD data of the mean blood flow velocity in the middle cerebral artery (MCA), of each of the 3 ramps.

6.2.4. Additional Information

In addition to the pupillometry and CVR assessments, all subjects completed basic physiological measurements and completed a questionnaire which collected information on demographics, physical and mental health, and their lifestyle. Additionally, a saliva sample was acquired from each subject to determine their apolipoprotein E (APOE) status, which is an important risk factor for Alzheimer's disease and other neurodegenerative diseases [56], [384], [385], [386]. Due to the limited size of the group, not all genetic and lifestyle factors could be assessed. However, data regarding sleep duration and quality was used for further analysis as

this was reported from all subjects, and since sleep has been shown to impact the PLR.

Three lifestyle questions relating to sleep were chosen to compare to the pupillometry parameters and to include as covariates in the PLR/PIPR and CVR relationship. These three questions evaluated subject's alertness on a scale of 1-9 using the Karolinska Sleepiness Scale (KSS) with labels on every step on the 9-point scale [387], their subjective sleep quality on a scale of 1-5 over the past week (1 – very poor, 2 – poor, 3 – fair, 4 – good, 5 – very good), and the average number of hours they slept in the past week (using half hour increments). The questions as they appeared in the subject questionnaire are shown in Appendix C.

6.2.5. Data Analysis

6.2.5.1. Pupillometry analysis

PyPlr data was processed using custom Python scripts. These allowed for the removal of low-confidence (< 0.8) measurements defined by the Pupil Labs software, filtering out high frequency noise using a Butterworth filter, and removing blinking artefacts using linear interpolation. The baseline pupil diameter was extracted before the light onset for each trial, as well as the pupil diameter at 7 s post-stimulus onset (7 s PIPR), expressed as a percentage of the baseline diameter. The red, blue, and overall (red – blue) 7 s PIPR values were averaged across trials. The red, blue, and overall 7 s PIPR values were compared between sexes using a one-way ANOVA test.

6.2.5.2. CVR analysis

CVR data was processed using custom Python scripts. The linear CVR values were averaged across all usable trials and compared between sexes using a one-way ANOVA test.

6.2.5.3. Comparison analysis

Each of the three questions relating to sleep quality and alertness were compared with each parameter of the PLR and PIPR using linear regression analysis (significance level $p < 0.05$, uncorrected), to identify any relationships independent of CVR between sleep and the PLR in this cohort.

To identify any statistically significant relationships between parameters of the PLR, PIPR, and CVR, linear regression analysis was performed between each pupillometry parameter and the linear CVR (significance level $p < 0.05$, uncorrected). This was then expanded to include age, sex, and three lifestyle questions assessing sleep quality and alertness as covariates in an ordinary least squares linear regression model.

6.3. Results

All 33 subjects completed the Neuroptics and PyPlr protocols and submitted a questionnaire which collected information about their lifestyle, demographics, and background. However, PyPlr data from 3 subjects was excluded due to high noise. For the CVR assessments, data from 9 subjects was excluded due to either high noise or subject difficulty with the gas challenge protocol, which did not allow for the full ramp sequence to be obtained. From our original cohort, 24 subjects (12F, age

range 18 – 70 years, with a mean of 32.7 ± 10.7 years at the time of acquisition) were left for further analysis.

6.3.1. Pupillometry Results

As outlined in Section 6.2.2, data were collected from both the right and left eyes of each participant for both pupillometry protocols. For the PyPlr pupillometry, many participants only had usable data from one eye due to noise in the protocols, and so the eye combining the highest average confidence and data completeness was selected individually for each participant. This was done, rather than choosing the same eye for all participants, to ensure that sufficient data was included for analysis.

For the NeurOptics, eight participants did not have data for the time to 75% recovery for their right eye in the light flash protocol, due to artefacts. Although the left eye was missing this data from 6 subjects (including one who also does not have dilation velocity data in the light flash protocol due to blinking artefacts), due to data completeness, the left eye of all subjects was used for further analysis for the NeurOptics protocol.

6.3.1.1. PyPlr Results

Table 6.1 shows the average values for the PyPlr protocol, for the entire group and separated by sex, with the p value included for the t-test between sexes.

***Table 6.1**: Post-illumination pupil response metrics from the PyPlr protocol*

	Female Mean ± Std Dev	**Male Mean ± Std Dev**	**Overall Mean ± Std Dev**	**p value**
7 s Red PIPR (% change from baseline)	-17.00 ± 3.86	-14.81 ± 3.48	-15.80 ± 3.74	0.18
7 s Blue PIPR (% change from baseline)	-37.62 ± 9.73	-45.06 ± 6.91	-41.68 ± 8.94	0.05*
Overall 7 s PIPR Difference (% change from baseline)	20.63 ± 8.62	30.24 ± 8.05	25.87 ± 9.48	0.01*

**: statistically significant differences across sexes*

Interestingly, there were statistically significant differences between sexes in both the 7 s blue PIPR ($p < 0.05$) and in the overall 7 s PIPR ($p = 0.01$), with the female group having lower magnitudes in both of these metrics, indicating a higher percentage recovery of the baseline pupil size in females. Although not statistically significant, the females displayed a trend toward a slightly higher magnitude of 7 s red PIPR compared to the male group ($p = 0.18$). However, these values are uncorrected for multiple comparisons, and once corrected they are no longer below the threshold for statistical significance.

6.3.1.2. NeurOptics Results

Tables 6.2 and 6.3 show the average values for the PLR metrics across both sexes, in the light and dark flash protocols respectively.

***Table 6.2**: Pupillary light response metrics for the light flash protocol, left eye*

	Female Mean ± Std Dev	Male Mean ± Std Dev	Overall Mean ± Std Dev	p value
Initial Diameter (mm)	6.28 ± 0.89	6.63 ± 1.06	6.46 ± 0.97	0.39
End Diameter (mm)	3.34 ± 0.59	3.77 ± 0.78	3.55 ± 0.71	0.14
Latency (s)	0.22 ± 0.03	0.22 ± 0.02	0.22 ± 0.02	0.77
Constriction Velocity (mm/s)	-3.22 ± 0.42	-3.01 ± 0.42	-3.11 ± 0.43	0.24
Max Constriction Velocity (mm/s)	-5.64 ± 0.88	-4.98 ± 1.19	-5.31 ± 1.08	0.13
Dilation Velocity (mm/s)	1.09 ± 0.52	0.91 ± 0.37	1.00 ± 0.45	0.35
Time to 75% Recovery (s)	5.21 ± 0.99	5.01 ± 0.95	5.12 ± 0.95	0.67
Amplitude of Constriction (mm)	2.94 ± 0.36	2.87 ± 0.40	2.90 ± 0.37	0.61

***Table 6.3**: Pupillary light response metrics for the dark flash protocol, left eye*

	Female Mean ± Std Dev	Male Mean ± Std Dev	Overall Mean ± Std Dev	p value
Initial Diameter (mm)	2.76 ± 0.53	3.10 ± 0.30	2.92 ± 0.46	0.08
End Diameter (mm)	3.80 ± 0.77	4.23 ± 0.49	4.01 ± 0.67	0.13
Latency (s)	0.37 ± 0.03	0.36 ± 0.04	0.36 ± 0.03	0.60
Dilation Velocity (mm/s)	1.49 ± 0.49	1.58 ± 0.39	1.53 ± 0.43	0.65
Amplitude of Dilation (mm)	1.04 ± 0.31	1.13 ± 0.25	1.09 ± 0.28	0.45

Contrary to the PyPlr PIPR protocol, there were no statistically significant differences observed between sexes in any of the NeurOptics PLR parameters across both the light and dark flash protocols, even without correcting for multiple comparisons. However, there was a weak trend towards differences in pupillary diameters between sexes – specifically, a decreased end diameter in the light flash protocol ($p = 0.14$) and decreased initial ($p = 0.08$) and end diameter ($p = 0.13$) in the dark flash protocol in the female group. Additionally, there was a weak trend toward an increased maximum constriction velocity magnitude in the light flash protocol for the females compared to the males ($p = 0.13$).

6.3.2. CVR Results

The linear CVR was the only CVR metric used for further analysis in this study. The mean value overall was 0.0333 mmHg/cm/s and there were no statistically significant differences across sexes ($p = 0.91$).

6.3.3. Comparison Results

6.3.3.1. Sleep and the PLR/PIPR

Without correcting for multiple comparisons, there was a statistically significant negative correlation between subjective sleep quality and maximum constriction velocity in the light flash protocol ($p = 0.048$), and a statistically significant negative correlation between the average hours of sleep and latency in the light flash protocol ($p = 0.029$). Correcting for multiple comparisons, these are no longer statistically significant. No other statistically significant relationships were identified between the three sleep questions and any parameter of the PLR or PIPR.

The full correlation results between the sleep and pupillometry parameters can be found in Appendix D.

6.3.3.2. CVR and PLR Comparisons

To compare linear CVR with each of the PLR and PIPR metrics, this analysis was conducted first without covariates, and subsequently with covariate analysis. The covariates included age, sex, and the same three sleep questions from the questionnaire as described in the previous section.

Table 6.4 shows the correlation between the PyPlr PIPR parameters and CVR both with and without covariates.

***Table 6.4**: Correlation between PyPlr PIPR parameters and CVR, with and without covariates*

	Correlation coefficient, no covariates	p value, no covariates	p value, with covariates
7 s Red PIPR (% change from baseline)	-0.15	0.51	0.30
7 s Blue PIPR (% change from baseline)	-0.16	0.47	0.80
Overall 7 s PIPR Difference (% change from baseline)	0.09	0.68	0.86

Tables 6.5 and 6.6 show the correlation between the NeurOptics PLR parameters and CVR both with and without covariates, in the light and dark flash protocols respectively.

***Table 6.5**: Correlation between light flash PLR parameters and CVR, with and without covariates, left eye*

	Correlation coefficient, no covariates	p value, no covariates	p value, with covariates
Initial Diameter (mm)	-0.19	0.38	0.38
End Diameter (mm)	-0.11	0.61	0.60
Latency (s)	-0.10	0.65	0.75
Constriction Velocity (mm/s)	0.13	0.54	0.90
Max Constriction Velocity (mm/s)	0.08	0.70	0.63
Dilation Velocity (mm/s)	-0.28	0.18	0.18
Time to 75% Recovery (s)	-0.27	0.22	0.42
Amplitude of Constriction (mm)	0.08	0.76	0.82

***Table 6.6**: Correlation between dark flash PLR parameters and CVR, with and without covariates, left eye*

	Correlation coefficient, no covariates	p value, no covariates	p value, with covariates
Initial Diameter (mm)	-0.26	0.24	0.56
End Diameter (mm)	-0.27	0.22	0.36
Latency (s)	0.00	0.99	0.47
Dilation Velocity (mm/s)	-0.26	0.24	0.18
Amplitude of Dilation (mm)	-0.23	0.30	0.20

6.4. Discussion

Interesting trends were identified with the subjects that remained for further analysis in this study. However, due to limitations in data quality and subject completion of protocols, particularly involving the TCD CVR values, nine subjects had to be removed from the analysis. Although this allowed for a preliminary analysis of trends, a larger subject cohort would enable further analysis, particularly involving additional covariates. Nonetheless, this cohort was specifically recruited with subjects from a variety of demographic and health backgrounds, which allowed for an investigation into the validity of the PLR/PIPR-CVR relationship not just in a healthy, relatively uniform group. The subject group spanned several ages, a mix of APOE genotypes, and some had additional lifestyle-related factors, such as smoking, low exercise levels, and high blood pressure, that placed them at a higher risk of developing Alzheimer's disease [5]. These are all factors that should be considered when assessing the PLR/PIPR-CVR relationship, as they may contribute as covariates or confounding factors to either the PLR, PIPR, or CVR.

The specific pupillometry protocols were chosen based on testing data from Chapter 3, feedback from Chapter 4, and additional pilot studies. The protocols with PyPlr enabled the use of chromatic pupillometry to identify specific contributions from melanopsin-dependent responses compared to rod/cone-dependent responses, and additionally to assess the PIPR rather than only considering the short-term pupillary responses of the PLR. The NeurOptics light flash protocol enabled a more classical measure of the pupillary light response, with the dark adaptation biasing this to use the rods over the cones [388], and the dark flash protocol enabling further

analysis and differentiation between the branches of the autonomic nervous system [16]. As such, the protocols chosen enabled consideration of not only the potential contributions of smooth muscle, but also the contributions of specific photoreceptors and branches of the autonomic nervous system.

The three questions relating to sleep quality were chosen based on previous literature and the ability to include this in the lifestyle questionnaire. Earlier versions of the questionnaire did not account for sleep quality or alertness, which meant that these could not be used in covariate analysis. The Karolinska Sleepiness Scale has been widely used and validated in research [387], [389] and was easily understood by subjects as they completed their questionnaire. Although subjective measures of sleep quality as well as an estimate of average sleep duration have their limitations and may vary in accuracy from individual to individual, these provided a preliminary investigation into sleep dynamics which could then be used to evaluate their interplay with the PLR/PIPR-CVR relationship.

When assessing the impact of sleep factors on all aspects of the PLR and PIPR assessed in this study, there were two statistically significant relationships identified. Subjective sleep quality was negatively correlated with maximum constriction velocity in the light flash protocol ($p = 0.048$), and the average hours of sleep was negatively correlated with latency in the light flash protocol ($p = 0.029$). Although not all statistically significant, there were also trends approaching significance in the light flash protocol – notably, subjective alertness on using the KSS had weak positive correlations with all parameters assessed in the light flash protocol aside from the time to 75% recovery, which had a weak negative correlation.

Additionally, subjective sleep quality had weak negative correlations with all parameters assessed in the light flash protocol, and the average hours of sleep were weakly negatively correlated with constriction velocity ($p = 0.087$) and maximum constriction velocity ($p = 0.19$). The positive trends with alertness are explained as the scale used to quantify alertness was the opposite of that to quantify sleep quality, with lower numbers corresponding to higher alertness, while both sleep quality and hours of sleep had higher numbers corresponding to better sleep. These relationships, although weak and without statistical significance when corrected, demonstrate how sleep quality may impact the autonomic nervous system, particularly the parasympathetic branch, as the trends were generally most pronounced in the dynamic responses of the constriction phase in the light flash protocol. Previous work has shown significant impacts of sleep directly on the pupillary light response [154], and how sleep deprivation can impact the autonomic nervous system [390]. Although no statistically significant relationships between sleep and the chromatic stimuli were identified in this study, other studies have found subjective sleepiness to be correlated with the post-stimulus pupil response to red light [391], as well as a significant association between the PIPR and decreased sleep quality in glaucoma patients [392]. These relationships also justify the inclusion of sleep as a covariate for further analysis with the PLR/PIPR-CVR relationship.

Females in this cohort were shown to have a statistically significantly smaller percent change from baseline in both the 7 s blue PIPR ($p < 0.05$, uncorrected) and overall 7 s PIPR ($p = 0.01$, uncorrected). Interestingly, the female response to red light, although not statistically significant, indicated a slightly lower percentage

recovery of the baseline pupil size in the 7 s red PIPR compared to men ($p = 0.18$), which contributed to the larger difference in overall 7 s PIPR in terms of percent recovery of baseline pupil size. Although these trends are no longer significant when accounting for multiple comparisons, this does identify a potential trend which should be further explored. In their study evaluating the transient pupillary light reflex to red and green stimuli in age-matched young adults, Fan *et. al.* did not observe significant sex differences in recovery speed [393]. Additionally, this supports the use of sex as a covariate in the analysis of the PLR/PIPR-CVR relationship conducted in this study.

Aside from sleep factors and sex, age was also added as a covariate between the PLR/PIPR and CVR. This was done since age has been shown to impact aspects of the PLR [394], [395], [396] and cerebral blood flow [397], [398], [399], and since this subject cohort had a large range of ages, it was important to consider this factor in the PLR/PIPR-CVR relationship as this had not been done previously.

Although previous work identified a statistically significant negative relationship between CVR and the latency of the dark flash protocol, this relationship was not statistically significant in this study. However, there were some changes made that could explain this. The previous study used a different protocol and metric for CVR assessment and had a smaller age range. Additionally, the current study did include subjects who had lifestyle risk factors for neurodegeneration, such as smoking history and high blood pressure, which could be confounding factors to the relationship and were excluded for this reason in the previous study. This difference

in results suggests that these factors are important in covariate analysis and should be considered as such in a larger cohort.

6.4.1. Limitations and Future Work

A limitation of this study was the cohort size, which limited the number of covariates that could be assessed without overfitting the data. With a larger cohort, additional lifestyle factors could be considered as covariates in the PLR/PIPR-CVR relationship, particularly those which are risk factors for neurodegenerative diseases as outlined in Chapter 2. Additionally, this would allow genetic information, which was already collected, to be used in the analysis, given that APOE status is an important factor in Alzheimer's disease risk [56], [81]. In the future, this information could be used not only in an ordinary least squares regression model, but in more advanced supervised machine learning algorithms to produce a comprehensive prediction of an individual's CVR and vascular health profile.

For the pupillometry protocols, there were additional limitations. The NeurOptics protocols have limitations which were previously discussed in Chapters 4 and 5, notably the frame rate of the device, and the calculation of dilation parameters in the light flash protocol. With the PyPlr protocol, although data was collected for both eyes, the resulting data often had significant noise, and most subjects only had data from one eye which was usable. Uncertainty in measurements using Pupil Core, an integral part of the PyPlr system, have been reported due to calibration and scaling [400], and these measurement errors can be difficult to address and interpret [401]. Some of this noise was also due to the Pupil Core glasses which did not fit each person's face well enough to get both pupils in the field of view at all gaze positions.

After collecting this data, it was noted that additional extenders could attach to the Pupil Core cameras to allow for more flexibility in the fit of the glasses, which could be incorporated into future data collection to get more reliable, binocular data.

Linear CVR was used in this analysis due to the estimation that subjects were within the linear region of the sigmoidal CVR response during the protocol. However, some subjects did display more sigmoidal responses which may have artificially reduced their measured value for linear CVR. This calculation of linear CVR from this specific gas challenge method was used as it showed high correlation with linear CVR assessed via functional magnetic resonance imaging using the same gas challenge, which in the future would enable further comparisons to other studies who use MRI as the gold standard for CVR assessment. However, the limitation of whether all subjects remain in the linear regime for CVR after inhaling 10% CO_2 remains unknown before testing. Future work could screen subjects in advance to ensure that the protocol can be handled, or a less intense gas challenge (such as only breathing up to 5% CO_2) could be used for calculating linear CVR instead.

6.5. Conclusion

This study explored the sleep-mediated relationship between the PLR, PIPR, and CVR in a diverse group of adults. Factors relating to sleep quality were shown to weakly impact some parameters of the pupillary light response, and sex differences were identified in the red and overall 7 s PIPR. Although no statistically significant relationships were observed in the PLR-CVR or PIPR-CVR relationship both with and without predefined covariates of age, sex, and sleep-related factors, the dilation velocity in both the light and dark flash protocol showed a weak relationship with

linear CVR. The lack of statistically significant observations in this study may be due to additional covariates mediating these relationships, such as lifestyle and genetic risk factors, which should be assessed in a larger cohort, where such analyses may be conducted.

Chapter 7: Conclusions and Future Work

This chapter aims to connect all the work presented in this thesis, summarize it, and provide direction and recommendations for future work and applications including potential clinical translations. It describes any planned work that has not been completed at the time of thesis submission, which includes a summary of the limitations and future work presented in each chapter, as well as a general summary of what will follow from this thesis. The research questions presented in Chapter 1 are answered with the findings of the thesis chapters, with the significance of the findings highlighted.

7.1. Summary of Findings

Neurodegeneration causes many biological changes, some of which may precede overt symptoms of cognitive decline. In previous studies and literature, both the pupillary light response and cerebrovascular reactivity have been identified as metrics that are impaired in neurodegeneration. This thesis aimed to examine, given the overlap between these two biologically relevant metrics, whether the pupillary light response is related to cerebrovascular reactivity and additionally, if this relationship can be used to classify risk for neurodegeneration.

To do this, several confounding factors such as established lifestyle factors for dementia, and other potential confounding factors less explored such as pregnancy history, were evaluated for their impact on the pupillary light response independently. Additionally, novel tools and methods were developed to investigate various parameters of the pupillary light response, and determine which, if any, had

the strongest relationship with cerebrovascular reactivity, neurodegeneration risk, and other covariate factors.

The introduction in **Chapter 1** provided a key overview of the topics discussed in this thesis, including the pupillary light response, cerebrovascular reactivity, neurodegeneration, and gold-standard imaging methods such as magnetic resonance imaging and transcranial Doppler ultrasound. It highlights the research questions that this thesis aimed to answer.

The literature review in **Chapter 2** showed that there are modifiable risk factors for dementia that affect the pupillary light response, which should be accounted for when considering the PLR-CVR relationship. It found that many factors including TBI, ocular and intracranial hypertension, alcohol consumption, depression, and diabetes are all AD risk factors that have demonstrated changes in the PLR. Other modifiable risk factors for AD are associated with changes to autonomic function but have not been studied directly with the PLR, and further research is necessary to investigate any potential relationships between these risk factors of AD and the PLR. Further, there is a comorbidity between some AD risk factors, and so further research is necessary to separate the individual impacts of these risk factors on the PLR. Ultimately, it was shown that the PLR is impaired not only in current AD patients but in certain at-risk groups, positioning the PLR as a potential candidate for risk screening.

The experiments in **Chapter 3** demonstrated some of the conditions and tools that were deemed most effective for evaluating various parameters of the PLR. Of note, the dark-adapted testing for the PLR was most effective for obtaining an effective

range of PLR parameters across a light flash protocol using the NeurOptics PLR-3000. The in-house PyPlr system was set up and tested using both white light and chromatic stimuli with various interstimulus intervals and was compared with the NeurOptics PLR-3000. For a simple white flash PLR protocol, the NeurOptics PLR-3000 was found to be superior to the PyPlr system overall, with more consistent and accurate data that can be compared to existing datasets. The in-house PyPlr system was, however, useful for evaluating the PIPR to red and blue light, so both pupillometers were used in subsequent chapters.

The pilot study conducted in **Chapter 4** used a steady state protocol to assess CVR and a light and dark flash protocol to assess the PLR. The study found a statistically significant negative relationship between a parameter of the pupillary light response (the dark flash latency) and cerebrovascular reactivity in a cohort of healthy adult participants. Further, this identified a potential relationship between smooth muscle function in the eye and the brain, with contributions from the autonomic nervous system and potentially other factors.

The pilot study conducted in **Chapter 5** was the first study to evaluate differences in the dynamic aspects of the pupillary light response in postpartum women and women who had never been pregnant, highlighting an understudied area of research which demonstrates the need to include sex-specific factors in biological analyses. Although no statistically significant differences were identified in this small cohort, there were some trends identified in some parameters of the PLR related to parasympathetic function that require further research.

Finally, the pilot study conducted in **Chapter 6** assessed the PLR/PIPR-CVR relationship. It demonstrated that the specific tools used to assess both the PLR and CVR have an impact on the PLR-CVR relationship, as the relationship between linear CVR and the PLR did not yield the same statistically significant relationship identified in Chapter 4. Additionally, it further suggested that a covariate analysis is required to fully describe this research, noting that sex and sleep-related factors influence the PLR and PIPR and may contribute to the relationship.

7.2. Future Work and Broader Applications

There is significant potential for future work that will come from the studies completed for this thesis, to address some of the limitations of this work and to expand the clinical applications and translations.

7.2.1. Larger Cohort Study

The work presented in this thesis involved small cohorts of participants, which were useful for identifying preliminary trends but did not allow for further applications using machine learning techniques to be used. A limitation of this thesis was the smaller study sample sizes, which limited the ability to investigate trends with all covariate factors or use machine learning for prediction due to the limited prediction power.

In the future, with more participants in studies such as the Eye-Brain Study and the Maternal Brain Study (both of which are ongoing at the time of thesis submission), there is potential to create a model that can take in an individual's PLR, genetic, lifestyle, and physiological data, and classify them based on their CVR and risk for Alzheimer's disease. Supervised machine learning will likely be the best method for

classifying the data into different risk categories, due to the nature of the categories and ultimately, the realistic size of the cohort being around 80-100 participants. The algorithm will be required to map from multiple inputs into multiple output classes – notably, risk classes based on the predicted CVR and AD risk. A proposed architecture for this classification algorithm is shown below in Figure 7.1.

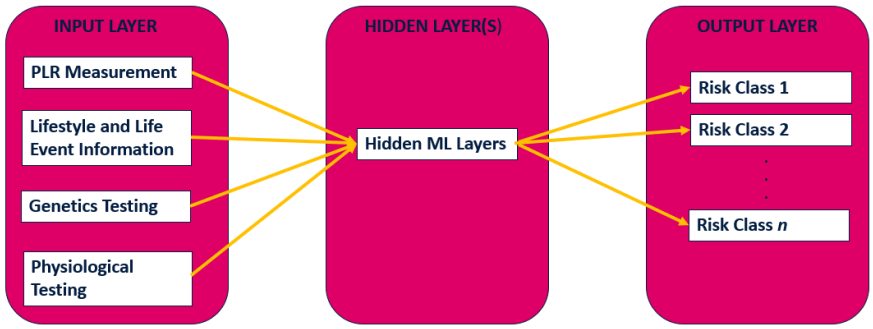

***Figure 7.1**: Proposed supervised machine learning architecture for a future classification algorithm, with outputs into risk classes based on CVR and AD risk.*

To ensure sufficient data is available to train the model, transfer learning approaches could be applied to make use of new datasets that arise with pupillary and demographic datasets. New pupillary datasets have recently emerged which include individuals at increased risk for AD due to lifestyle risk factors such as diabetes [402].

It will be necessary to choose a machine learning model to classify data. Within the realm of supervised machine learning, there are three model types that will likely suit the aims of this future proposed classification model: random forests, artificial neural networks, and support vector machines. Each of these models could be assessed with data collected through the Eye-Brain, Maternal Brain, and subsequent PLR-CVR studies to determine which is most suitable for the PLR and CVR-based AD classifying algorithm. This model could be evaluated using future data from these

studies, and after this validation, genetic, lifestyle, and physiological factors could be assessed for their contributions to the PLR-CVR relationship. Factors that are redundant to the classification algorithm may be noted and removed from the model, which could save time in future studies when assessing for these factors using questionnaires or physiological assessments.

7.2.2. Web or smartphone application to assess PLR

There are currently pupillometers that use either a laptop camera or smartphone camera to perform PLR measurements, which increases the accessibility of pupillometry in a variety of settings. Examples include applications on both the web for real-time pupillometry [403], and through smartphone applications using phone cameras app for pupillometry [404], [405]. However, many of these online pupillometers are more difficult to use and cannot easily differentiate between the iris and pupil of darker coloured eyes. Because of these shortfalls, there is potential to improve these designs and to create a new and improved web or smartphone application to assess the PLR, which could integrate some of the code used in the GitHub repositories included in this thesis or other open-source code available. With an app to assess the PLR, there would be opportunities for data collection on a much larger scale. The application could then have an option to consent to record lifestyle information in addition to assessing their PLR, and this data could be sent to a secure research database to be shared with researchers across the globe.

7.2.3. Clinical tool

As mentioned in some chapters, there were some limitations with the hardware and equipment used, which could be improved in future studies – notably, the frame rate

of the pupillometer and the requirement for blink removal. There is a possibility that the work done throughout this project could be used in a clinical setting as an inexpensive diagnostic medical device, which could incorporate some of these improvements to a novel pupillometry system. Although the NeurOptics PLR-3000 outperformed the current PyPlr system in white flash protocols shown in Chapter 3, the PyPlr system had not been tested with a dark flash protocol and could potentially be used for this and other protocols with some improvements. The dark flash protocol has been shown to be important in identifying trends between the PLR and CVR, and thus a future clinical tool should support this protocol in addition to a regular, white flash protocol, and potentially chromatic pupillometry as demonstrated in this thesis.

A novel classification model could be integrated into the pupillometer, which could be used for routine monitoring of an individual's PLR and their predicted CVR and associated risk for AD in a clinical setting. This device could be available to clinicians for screening purposes, and if the patients and hospitals agreed, this clinical data could further be used to feed back into the model as well as a secure database to strengthen the screening and predictive quality. The PLR, in conjunction with the demographic, lifestyle, and genetic factors deemed necessary to predict CVR, could screen for those who should get a confirmatory MRI to identify any changes to their cerebral vasculature, and individuals could then be sent to targeted interventions based on their individual risk profiles.

7.2.4. Inclusion of the PLR in Large-Scale Databases

As demonstrated in this thesis, there are many physiological, environmental, and other factors that influence pupil size and the pupillary light response. Although it may be seen as difficult to disentangle the contributions of these factors on the pupillary light response, conversely this demonstrates how much information is held in the pupillary light response. This strength of the PLR could be made use of in large-scale health informatics programmes and databases, such as the UK Biobank, enabling researchers to gain insight into the neurological, visual, and physiological inputs to the PLR and how these may manifest in other diseases, including Alzheimer's disease and neurodegeneration. With larger cohorts, such as those available in these large-scale databases, researchers could make use of machine learning and other tools for risk factor analysis and prediction.

7.3. Thesis Conclusion

Alzheimer's disease and dementia are rapidly increasing around the world, and the current aim is to prevent the onset of the disease. The pupillary light response has been shown to be impaired in current AD subjects, and prior to this work, it was unknown if it could be used as a tool in at-risk groups to predict AD risk. Cerebrovascular reactivity has been shown as a likely candidate for aiding in dementia prediction. Combining these biomarkers of AD, this thesis presents an initial exploration of the relationship between the PLR and AD risk, mediated by a key component of brain vascular health, cerebrovascular reactivity.

Below, I present a summary of the answers to the main questions of this thesis:

1. *How do established Alzheimer's disease risk factors influence the pupillary light response?*

From the literature review in Chapter 2, key relationships between AD risk factors and the PLR were identified. TBI, ocular and intracranial hypertension, alcohol consumption, depression, and diabetes are all AD risk factors that have demonstrated changes in the PLR, which should be accounted for when considering the PLR and its ability to be used as a potential AD screening tool.

Chapter 5 demonstrated that sex-specific factors, such as pregnancy history, may impact parameters of the PLR – in particular, trends toward a decreased dilation velocity in a dark flash PLR protocol was observed in the postpartum group compared to women who had never been pregnant, indicating a potential impact of pregnancy on sympathetic innervation in the postpartum period. Additionally, when including demographic and physiological covariates including age, BMI, blood pressure, and resting heart rate, parameters of the PLR (light and dark-adapted pupil size, light and dark flash latency) were also significantly impacted by postpartum status, indicating that both static and dynamic components of the pupillary light response may be mediated by age and physiological metrics that may also relate to AD risk. Due to sex differences in the manifestation and risk of dementia and demonstrated changes in risk for AD associated with pregnancy, this demonstrates that pregnancy history is an important consideration impacting both the PLR and AD risk. Further work should be done to investigate other sex-specific factors and how they may impact the PLR and AD risk.

This question contains areas for future investigation. Notably, many risk factors associated with AD had inconclusive findings when investigating their relationship between the factor and the PLR. Hearing loss, smoking, and genetic factors have had associations with changes to the parasympathetic activity, which could indicate impaired PLRs, however further research should be done to confirm this hypothesis. No conclusions could be drawn between education, social isolation, obesity, and physical inactivity and the PLR, mainly due to a lack of literature, so more research should be done to investigate any potential relationships. Further, there is a comorbidity between some AD risk factors, and so further research is necessary to separate the individual impacts of these risk factors on the PLR. Finally, there are more risk factors that have been identified for Alzheimer's disease since the publication of the literature review in Chapter 2, such as vision loss and high LDL cholesterol [5], and more unknown risk factors, which should be further explored.

2. *What parameters of the pupillary light response relate most strongly to cerebrovascular reactivity?*

From the pilot study conducted in Chapter 4, it was shown that the latency of the dark flash response had the strongest relationship with steady state CVR, with a negative linear relationship being observed in healthy adults. Although statistical significance was only observed when uncorrected for multiple comparisons, this demonstrated the potential applications of the dark flash PLR protocol for identifying trends with CVR, as well as for general use in broader pupillometry applications.

Chapter 6 demonstrated that white light generally had stronger relationships with linear CVR than the chromatic stimuli. Although no statistically significant relationships were identified between any of the PLR parameters across the light flash, dark flash, and chromatic pupillometry protocols, dilation velocity in both the light and dark flash protocols displayed a weak negative linear trend with linear CVR. There was no change to the strength of this relationship when including factors relating to sleep, sex, and age as covariates in the light flash protocol, but when including these in the dark flash protocol, the trend became stronger. This once again highlights some differences observed between the light and dark flash protocols and supports the use of both protocols for assessing the PLR-CVR relationship.

Additional work is required to confirm these findings to fully answer this research question. Studies conducted in a larger, more diverse cohort will enable further clarification and confirmation of results. All three pupillometry protocols presented in Chapter 6 that were developed from initial work in Chapter 3 should be used in these future studies, due to the differing relationships observed and their interplay with other covariate factors.

3. *How do genetic, lifestyle, environmental, and demographic factors influence the relationship between the pupillary light response and cerebrovascular reactivity?*

Discrepancies identified between Chapters 4 and 6 highlight how additional factors may mediate the PLR-CVR relationship. The trends that were identified in the pilot study from Chapter 4 which only included healthy adults were not the

same observed the larger, more diverse cohort, which included adults with elevated genetic and lifestyle risk factors for Alzheimer's disease, shown in Chapter 6. Further, when including covariates such as sex, age, and factors relating to sleep duration and quality, the relationships observed between factors of the PLR and linear CVR changed – in some cases the relationships were strengthened, and in others they weakened.

Due to the limited cohort sizes within the scope of this research, in-depth analyses could not be conducted on the impact of all lifestyle, genetic, environmental, and demographic factors. Despite this limitation, factors including sex and pregnancy status, age, and sleep were included for further analysis in Chapters 4, 5, and 6. With the pilot study comparing the PLR and CVR in healthy adults in Chapter 4, no sex differences were identified in any parameter of the PLR or CVR assessed. Interestingly, when incorporating chromatic pupillometry methods using the PyPlr system in Chapter 6, sex differences were observed in the red and overall 7s PIPR, which may contribute to the PIPR-CVR relationship and requires further investigation. One sex-specific factor, postpartum status, demonstrated a weak impact on dilation velocity in the dark flash PLR protocol without controlling for covariates, and several static and dynamic parameters of the PLR were impacted by postpartum status when including age and physiological covariates in Chapter 5. This motivates further research investigating pregnancy and other sex-specific factors and their impact not only on various parameters of the PLR, but on the PLR-CVR relationship – in particular, incorporating chromatic pupillometry as this was not included within the scope of Chapter 5.

Self-reported sleep quality, duration, and reported alertness, influenced parameters of the PLR as expected from previous literature, which further motivated the inclusion of sleep duration and quality into a covariate analysis used in Chapter 6.

With a larger cohort, regression models incorporating the full demographic, genetic, and lifestyle information collected in both the Eye-Brain and Maternal Brain study questionnaires could be used to disentangle the impact of these factors on the PLR-CVR relationship. At the time of thesis submission, these additional factors were not included as covariates in the PLR-CVR relationship to avoid generalisations outside of the scope of the data, and to avoid overfitting the data in the small cohorts. In a larger cohort, factors including the ε4 allele, lifestyle factors influencing dementia risk as outlined in Chapter 2, and demographic factors including ethnicity should be included in a covariate analysis and in any predictive model that is used to infer CVR from the PLR.

This research is significant in many ways. If the PLR is shown to be an important factor in classifying CVR-based risk categories, clinical trials would benefit from using this as an inexpensive, quick, and easy means of evaluating many patients. Further, this would allow for the creation of a new clinical device for assessing the PLR in conjunction with genetic, lifestyle, and life-event factors, which would provide an opportunity for use in routine medical check-ups. Additionally, the methods used in this research, if successful, would make screening for risk of Alzheimer's disease and dementia more accessible to individuals who may not ordinarily have the financial or material means otherwise. If specific lifestyle factors are associated with

increased risks of developing conditions such as dementia, this could aid in their intervention across the globe.

Appendix A: Spectral Power Distributions of Light Stimuli

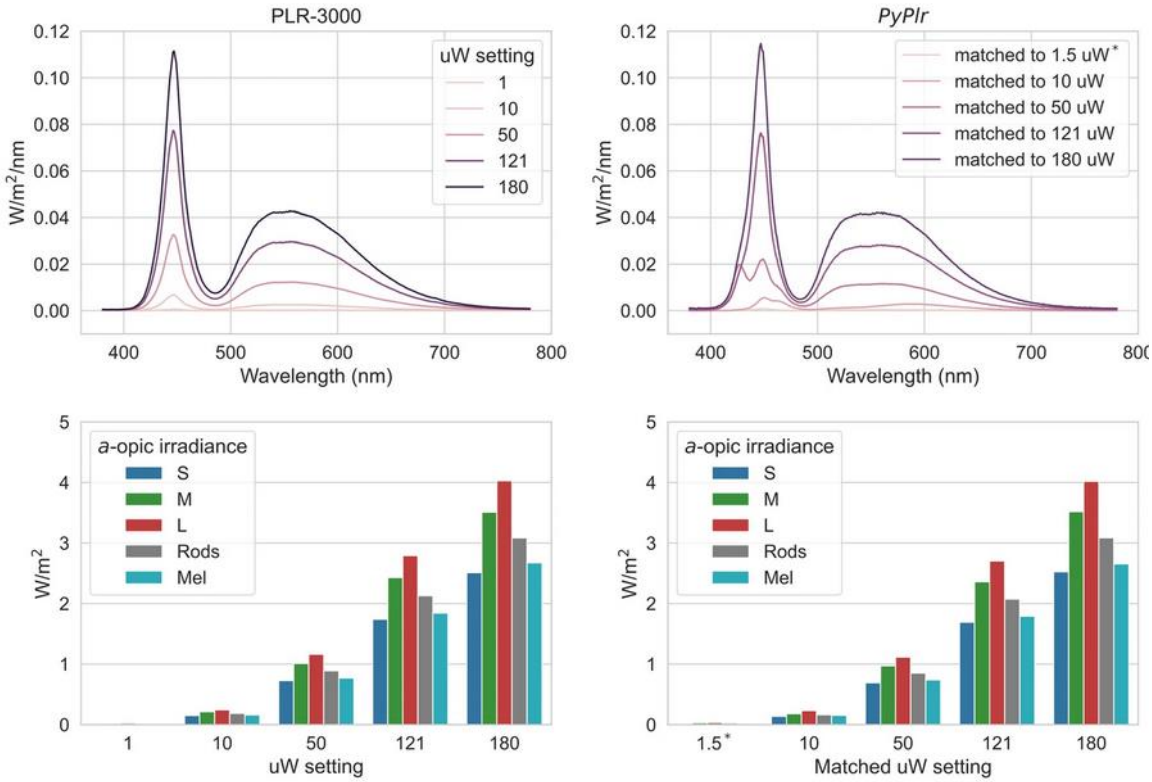

***Figure A.1**: Spectral power distributions and α-opic irradiances of the stimuli from the NeurOptics PLR-3000 and PyPlr devices, using a white light protocol. The curves at 50 μW represent the stimuli used in Chapters 3 (both devices), 4 (NeurOptics only), 5 (NeurOptics only), and 6 (NeurOptics only). Retrieved from Martin* et al. *[299].*

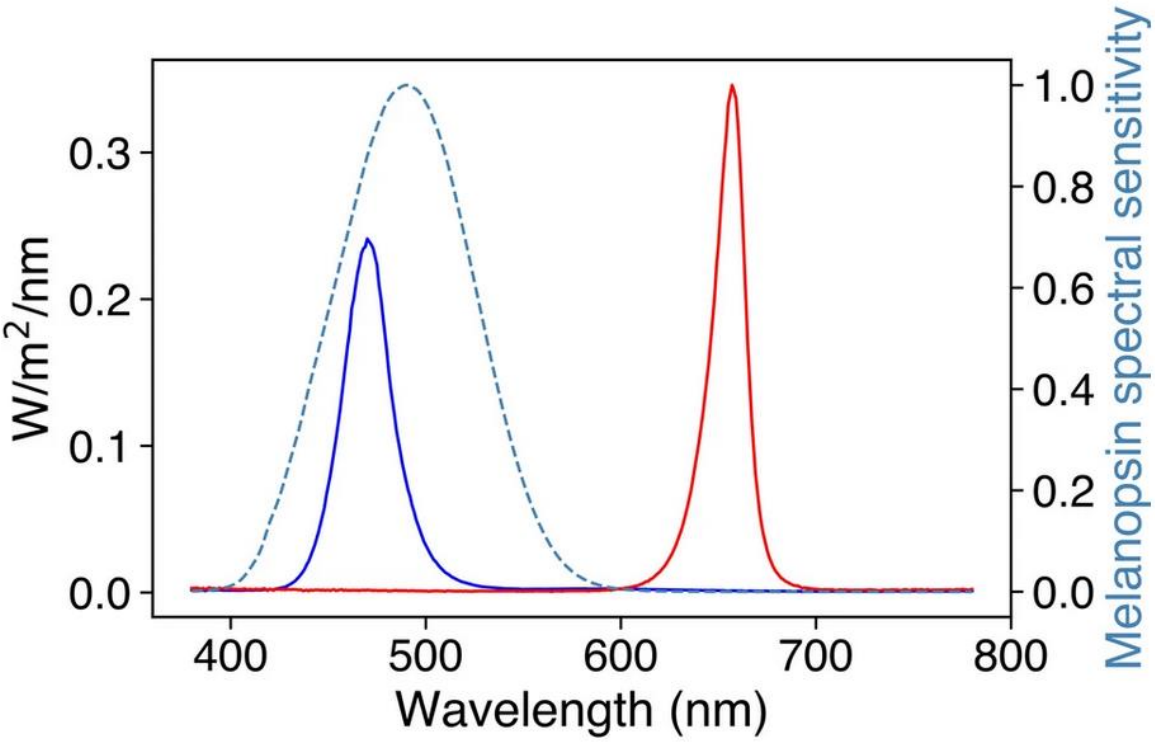

***Figure A.2**: Spectral power distribution of the stimuli from the PyPlr device, for the blue and red protocol, shown relative to melanopsin peak sensitivity. This stimuli was used in Chapters 3 and 6. Retrieved from Martin* et al. *[299].*

Appendix B: ANCOVA Analysis for PLR Metrics in Postpartum and Control Groups (Chapter 5 Appendix)

B.1. Light Flash Protocol

***Table B.1**: Initial Diameter ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	3.192923	1	8.695941	**0.02565**
Age	0.4807	1	1.309189	0.296127
BMI	0.000236	1	0.000643	0.980595
Postpartum Months	0.148133	1	0.40344	0.548759
Systolic Blood Pressure	0.00402	1	0.010948	0.920076
Diastolic Blood Pressure	0.154445	1	0.420632	0.540634
Resting Heart Rate	0.136201	1	0.370945	0.564822
Residual	2.203044	6		

***Table B.2**: End Diameter ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	2.782329	1	17.92824	**0.005475**
Age	1.077655	1	6.943991	**0.038792**
BMI	0.049379	1	0.318182	0.593145
Postpartum Months	0.001625	1	0.010469	0.921839
Systolic Blood Pressure	0.031408	1	0.20238	0.668603
Diastolic Blood Pressure	0.414885	1	2.673358	0.153158
Resting Heart Rate	0.327992	1	2.113455	0.196236
Residual	0.931155	6		

***Table B.3**: Latency ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.007246	1	11.39576	**0.014936**
Age	2.29E-05	1	0.036002	0.855767
BMI	9.41E-05	1	0.148022	0.713701
Postpartum Months	9.95E-06	1	0.01565	0.90453
Systolic Blood Pressure	0.000411	1	0.647079	0.451846
Diastolic Blood Pressure	0.000435	1	0.684683	0.439641
Resting Heart Rate	4.75E-05	1	0.074701	0.793775
Residual	0.003815	6		

***Table B.4**: Constriction Velocity ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.010415	1	0.029659	0.868929
Age	0.26632	1	0.758383	0.417312
BMI	0.002854	1	0.008128	0.931096
Postpartum Months	0.209378	1	0.596233	0.469331
Systolic Blood Pressure	0.118408	1	0.337183	0.582597
Diastolic Blood Pressure	0.041284	1	0.117562	0.743387
Resting Heart Rate	0.079478	1	0.226326	0.651088
	2.107012	6		

***Table B.5**: Maximum Constriction Velocity ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	1.39E-06	1	1.65E-06	0.999017
Age	0.421007	1	0.49964	0.506169
BMI	0.023212	1	0.027548	0.873628
Postpartum Months	0.006475	1	0.007684	0.933
Systolic Blood Pressure	0.005672	1	0.006731	0.93728
Diastolic Blood Pressure	1.01E-05	1	1.20E-05	0.997354
Resting Heart Rate	0.831542	1	0.986853	0.358871
Residual	5.05572	6		

***Table B.6**: Dilation Velocity ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.023212	1	0.18035	0.68588
Age	0.06313	1	0.490498	0.509937
BMI	0.016561	1	0.128677	0.732092
Postpartum Months	0.075026	1	0.582929	0.474108
Systolic Blood Pressure	0.084106	1	0.653479	0.449727
Diastolic Blood Pressure	0.026607	1	0.206728	0.66533
Resting Heart Rate	0.025179	1	0.19563	0.673769
Residual	0.772233	6		

***Table B.7**: Time to 75% Recovery ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.742282	1	2.292699	0.180755
Age	0.128623	1	0.397279	0.551732
BMI	0.010434	1	0.032229	0.863436
Postpartum Months	0.130855	1	0.404173	0.548408
Systolic Blood Pressure	0.475934	1	1.470026	0.270911
Diastolic Blood Pressure	0.08921	1	0.275543	0.618459
Resting Heart Rate	1.163007	1	3.5922	0.106862
Residual	1.942554	6		

***Table B.8**: Constriction Amplitude ANCOVA Table, Light Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.014124	1	0.069227	0.801267
Age	0.118871	1	0.582636	0.474214
BMI	0.042788	1	0.20972	0.663103
Postpartum Months	0.11873	1	0.581949	0.474464
Systolic Blood Pressure	0.057901	1	0.283796	0.613367
Diastolic Blood Pressure	0.063062	1	0.309091	0.598343
Resting Heart Rate	0.041474	1	0.203281	0.667921
Residual	1.224134	6		

B.2. Dark Flash Protocol

***Table B.9**: Initial Diameter ANCOVA Table, Dark Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	1.087532	1	25.51476	**0.002331**
Age	0.315589	1	7.404098	**0.034595**
BMI	0.020694	1	0.485506	0.512017
Postpartum Months	0.008319	1	0.195163	0.674131
Systolic Blood Pressure	0.009738	1	0.228459	0.649585
Diastolic Blood Pressure	0.179506	1	4.211432	0.085982
Resting Heart Rate	0.0772	1	1.811205	0.226987
Residual	0.255742	6		

***Table B.10**: End Diameter ANCOVA Table, Dark Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	1.198613	1	9.484021	**0.021674**
Age	0.212637	1	1.682493	0.242235
BMI	0.002308	1	0.018259	0.896931
Postpartum Months	0.002961	1	0.023426	0.883373
Systolic Blood Pressure	0.004358	1	0.034484	0.858799
Diastolic Blood Pressure	0.14275	1	1.129507	0.328773
Resting Heart Rate	0.041288	1	0.326691	0.58837
Residual	0.758294	6		

***Table B.11**: Latency ANCOVA Table, Dark Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.010722	1	9.827963	**0.020197**
Age	0.000431	1	0.394805	0.552935
BMI	0.003839	1	3.518861	0.109783
Postpartum Months	0.001105	1	1.013126	0.353008
Systolic Blood Pressure	1.67E-05	1	0.015324	0.905525
Diastolic Blood Pressure	0.000306	1	0.280591	0.615333
Resting Heart Rate	0.001793	1	1.643257	0.247182
Residual	0.006546	6		

***Table B.12**: Dilation Velocity ANCOVA Table, Dark Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.001746	1	0.016729	0.901316
Age	0.028221	1	0.270321	0.621734
BMI	0.208048	1	1.992822	0.207743
Postpartum Months	0.004314	1	0.041326	0.845629
Systolic Blood Pressure	0.005338	1	0.051132	0.828612
Diastolic Blood Pressure	0.036955	1	0.353982	0.573605
Resting Heart Rate	0.098007	1	0.938775	0.370012
Residual	0.626392	6		

***Table B.13**: Dilation Amplitude ANCOVA Table, Dark Flash Protocol*

	Sum sq.	df	F	PR(>F)
Group	0.0027	1	0.057443	0.818563
Age	0.01013	1	0.215494	0.658861
BMI	0.036823	1	0.783328	0.410188
Postpartum Months	0.001354	1	0.028801	0.870817
Systolic Blood Pressure	0.001067	1	0.022696	0.885188
Diastolic Blood Pressure	0.002103	1	0.04474	0.839485
Resting Heart Rate	0.005573	1	0.118562	0.742347
Residual	0.282047	6		

Appendix C: Questions Relating to Sleep Quality and Alertness (Chapter 6 Appendix 1)

Below are the questions relating to sleep quality and alertness from the Eye-Brain Study lifestyle questionnaire given to all participating subjects, used in the covariate analysis of Chapter 6.

2.5 Sleep quality and timing		
2.5.1	How alert do you feel right now? (Select one)	1: Extremely alert 2: Very alert 3: Alert 4: Rather alert 5: Neither alert nor sleepy 6: Some signs of sleepiness 7: Sleepy, but no effort to keep awake 8: Sleepy, some effort to keep awake 9: Very sleepy, great effort to keep awake, fighting sleep
In the **past 7 days**…		
2.5.2	My sleep quality was (Select one)	Very poor Poor Fair Good Very good Prefer not to answer
2.5.3	On average, how many hours did you sleep per night?	_____ hours Do not know Prefer not to answer

Appendix D: Sleep and PLR/PIPR Correlation Tables (Chapter 6 Appendix 2)

***Table D.1**: Correlation between sleep parameters and PIPR parameters*

Sleep Factor	PyPlr Parameter	Pearson Correlation Coefficient	p value
Alertness	7 s Red PIPR	-0.29	0.18
Alertness	7 s Blue PIPR	0.20	0.37
Alertness	Overall 7 s PIPR Difference	-0.31	0.17
Sleep Quality	7 s Red PIPR	0.26	0.25
Sleep Quality	7 s Blue PIPR	0.06	0.79
Sleep Quality	Overall 7 s PIPR Difference	0.04	0.85
Sleep Hours	7 s Red PIPR	0.32	0.15
Sleep Hours	7 s Blue PIPR	-0.04	0.86
Sleep Hours	Overall 7 s PIPR Difference	0.16	0.47

***Table D.2**: Correlation between sleep parameters and light flash PLR parameters*

Sleep Factor	Neuroptics Parameter	Pearson Correlation Coefficient	p value
Alertness	Initial Diameter	0.18	0.41
Alertness	End Diameter	0.19	0.37
Alertness	Latency	0.28	0.19
Alertness	Constriction Velocity	0.12	0.59
Alertness	Max Constriction Velocity	0.04	0.86
Alertness	Constriction Amplitude	0.10	0.63
Alertness	Dilation Velocity	0.32	0.14
Alertness	Time to 75% Recovery	-0.18	0.48
Sleep Quality	Initial Diameter	-0.17	0.43
Sleep Quality	End Diameter	-0.20	0.36
Sleep Quality	Latency	-0.15	0.48
Sleep Quality	Constriction Velocity	-0.29	0.17
Sleep Quality	Max Constriction Velocity	-0.41	**0.05**
Sleep Quality	Constriction Amplitude	-0.06	0.77
Sleep Quality	Dilation Velocity	-0.05	0.81
Sleep Quality	Time to 75% Recovery	-0.07	0.79
Sleep Hours	Initial Diameter	0.15	0.50
Sleep Hours	End Diameter	0.08	0.72
Sleep Hours	Latency	-0.45	**0.03**
Sleep Hours	Constriction Velocity	-0.36	0.09
Sleep Hours	Max Constriction Velocity	-0.28	0.19
Sleep Hours	Constriction Amplitude	0.23	0.28
Sleep Hours	Dilation Velocity	-0.01	0.96
Sleep Hours	Time to 75% Recovery	0.05	0.85

***Table D.3**: Correlation between sleep parameters and dark flash PLR parameters*

Sleep Factor	Neuroptics Parameter	Pearson Correlation Coefficient	p value
Alertness	Initial Diameter	0.13	0.55
Alertness	End Diameter	0.08	0.72
Alertness	Latency	-0.03	0.88
Alertness	Dilation Velocity	0.03	0.90
Alertness	Dilation Amplitude	-0.02	0.91
Sleep Quality	Initial Diameter	0.09	0.69
Sleep Quality	End Diameter	0.02	0.93
Sleep Quality	Latency	-0.22	0.31
Sleep Quality	Dilation Velocity	-0.06	0.77
Sleep Quality	Dilation Amplitude	-0.10	0.65
Sleep Hours	Initial Diameter	0.09	0.70
Sleep Hours	End Diameter	0.17	0.44
Sleep Hours	Latency	-0.21	0.34
Sleep Hours	Dilation Velocity	0.30	0.16
Sleep Hours	Dilation Amplitude	0.26	0.23

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